Genetic constraint, non-independence of traits and consequences of adaptive evolution in acridid grasshoppers

Dissertation

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“Darwin’s real revolution consisted in the epistemological reorientation that had to occur before the variational mechanism could even be formulated. It was a change in the object of study from the average or modal properties of groups to the variation between individuals within them. That is, *variation itself* is the proper object of biological study, for it is the ground of biological being. Without it, there would have been no evolution and therefore no living biological world [...]**”**

-**Richard C. Lewontin**, *Darwin’s Revolution, The New York Review of Books: June 16, 1983*

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Summary

Evolutionary trajectories of quantitative traits in phenotypic space are primarily influenced by the underlying genetic architecture of such traits. Genetic architecture can be studied at various levels, and one such way is to work with the additive genetic variances and covariances. Traits do not occur in isolation, and evolutionary change which is the change in trait values across generations usually occurs in multivariate trait space. Such non independent inheritance of traits occurs due to the existence of genetic correlation which arises due to pleiotropy or linkage disequilibrium. The resulting genetic covariation strongly affects the response to selection of traits, and can be statistically summarized by the additive genetic variance covariance matrix, **G**. **G** can change due to strong selection and/or drift, but can be stabilized under certain conditions like pleiotropic mutations, large population size etc. for certain categories of trait. On a long evolutionary time scale, **G** is bound to change and hence can diverge among populations and among species. Historically, quantitative genetics was predominated by the estimation of additive genetic variance, and all other external influences were categorized as environmental component. But partitioning phenotypic variance into these broad components largely ignores finer perspectives. For example, trait expression can be influenced by an interacting conspecific which from the perspective of a focal individual is part of the environment. If the interaction effects are heritable then such effects can evolve along with direct genetic effects. This heritable influence of the interacting partner on the focal individual's phenotype is called indirect genetic effect or IGE, and can be as important as direct genetic effects in shaping the response to selection of such interacting phenotypes.

My dissertation has two major themes, the estimation and comparison of additive genetic variance covariance matrices for morphological and song traits in grasshoppers, and the estimation of male indirect genetic effects on fecundity traits in a grasshopper under a potential sexual conflict scenario.

In the first two chapters I have studied the evolution of shape and orientation of **G** for morphological and song traits. Morphology is a set of traits which are conserved and are shown to have stable **G** matrices in comparison to behavioral or life history traits. On the other hand, song traits are behavioral male ornamental traits which are used in mate recognition as well as in attracting females and are presumably under sexual selection. Using a comparative quantitative genetic approach, I have estimated and compared 10 x 10 **G** matrices for five morphological traits in males and females in three species of closely related grasshoppers, and 5 x 5 **G** matrices for five song traits in two species of grasshoppers using a half-sib breeding design. I also estimated genetic correlations between traits as a measure of genetic constraint, and cross-sex correlations between male and female morphological traits as a measure of constraint to the evolution of sexual dimorphism. I scrutinized whether the overall genetic covariance structure aligned with the phenotypic divergence

in the three species. In the third chapter of my thesis, I estimated male IGEs which can manipulate female reproductive traits to maximize fitness. Males and females have conflicting evolutionary interests over trade-offs between current and future reproduction leading to a probable situation of sexually antagonistic coevolution. I measured five female fertility traits and estimated male indirect and female direct genetic effects using a half-sib breeding design.

I found that the **G** matrices of highly conserved morphological traits are different in shape and orientation and have diverged among species. The alignment of genetic variances with the main divergence axis was prominent only in the youngest species pair, *Chorthippus biguttulus* and *Gomphocerippus rufus*. Moreover, the divergence in **G** matrices carried a phylogenetic signal, which means the most distant species, *Pseudochorthippus parallelus*, showed the most dissimilar **G** matrix. Wing length turned out to be the most important trait along which the **G** matrices have diverged. The degree of genetic constraint was moderate (average 0.34) between traits and the cross-sex correlations were large (average 0.54), especially for lobe height and eye height. I found that the **G** matrices of male ornamental song traits show substantial differences and have diverged. The song traits show overall high heritabilities with the highest for syllable duration (0.50) and strophe duration (0.54) in *C. biguttulus* and strophe duration (0.30) in *G. rufus*. Maximum amplitude, a trait previously known to be under sexual selection in *C. biguttulus*, show low heritability (0.26) as predicted by theory. I also found strong negative genetic (-0.65) as well as phenotypic (-0.48) correlation between amplitude and strophe duration indicating amplitude increases with decrease of song duration. In the third chapter of my dissertation, I found low levels of male IGE on female fecundity traits, which are not significantly different from zero. Though initially after mating, the IGEs were high at least for egg pod length, number of egg pods and number of eggs per pod, they tapered with time. The female direct genetic effects were low to moderate.

In essence, the results indicate that even under a promising sexual conflict scenario, the phenotypic evolution of female reproductive traits will be influenced mostly by direct selection on female additive genetic variance rather than indirectly on male indirect genetic effects. In the first two chapters, the results demonstrate that **G** matrices have diverged in shape and orientation for both conserved morphological traits as well as for variable song traits. The differentiation in morphology has been along wing length, and the divergence bears a phylogenetic signal. Overall, evolutionary trajectories of both the song and morphology seem to be directly influenced by selection.

Zusammenfassung

Die Evolution quantitativer Merkmale wird entscheidend mitbestimmt durch die zugrundeliegende genetische Architektur. Es gibt unterschiedliche Ansätze, die genetische Architektur zu untersuchen, und einer davon ist die Analyse von additiv-genetischen Varianzen und Kovarianzen. Da Merkmale nicht isoliert voneinander evolvieren, findet evolutionäre Veränderung – also die Veränderung der Merkmalswerte über Generationen hinweg – stets in einem multivariaten Merkmalsraum statt. Die gekoppelte Vererbung von Merkmalen zeigt sich in genetischen Kovarianzen, die von pleiotropen Effekten oder Kopplungsungleichgewichten herrühren. Die genetische Kovariation hat einen starken Einfluss auf die Selektionsantwort und kann statistisch durch eine additiv-genetische Varianz-Kovarianz-Matrix **G** zusammengefasst werden. Die additiv-genetische Varianz-Kovarianz-Matrix **G** kann sich aufgrund starker Selektion und/oder Drift verändern oder durch pleiotrope Mutationen, große Populationsgrößen und andere Rahmenbedingungen stabilisiert werden. Auf einer evolutionären Zeitskala evolvieren **G**-Matrizen daher selbst und können sich zwischen Populationen und zwischen Arten auseinanderentwickeln.

Historisch gesehen wurde die quantitative Genetik durch die Schätzung der additiv-genetischen Varianz dominiert, während alle anderen äußeren Einflüsse als Umweltkomponenten gefasst wurden. Allerding lässt der Fokus auf die Aufteilung der phänotypischen Varianz in genetisch versus umweltbedingt viele Details außer Acht. So kann die Merkmalsausprägung beispielsweise auch durch Interaktionspartner beeinflusst werden. Aus der Perspektive des speziellen Individuums sind Interaktionspartner Teil der Umwelt. Allerdings können Interaktionseffekte mit direkten genetischen Effekten ko-evolvieren, wenn die Interaktionseffekte vererbbar sind. Dieser vererbbare Einfluss des Interaktionspartners auf den Phänotyp des Fokusindividuums wird als indirekter genetischer Effekt (IGE) bezeichnet und kann für die Selektionsantwort interagierender Phänotypen ebenso wichtig sein wie direkte genetische Effekte.

Meine Dissertation hat zwei Hauptthemen, der Abschätzung und dem Vergleich additiv-genetischer Varianz-Kovarianz-Matrizen für morphologische und Gesangsmerkmale bei Heuschrecken und die Abschätzung der indirekten genetischen Effekte auf Fruchtbarkeitsmerkmale bei einer Heuschrecke unter einem potentiellen sexuellen Konfliktszenario.

In den ersten beiden Kapiteln habe ich die Entwicklung der Struktur von **G**-Matrizen für morphologische und Gesangsmerkmale untersucht. Die Morphologie besteht Merkmalen, die bei Heuschrecken vergleichsweise konserviert sind und sich im Vergleich zu Verhaltens- oder Fitnessmerkmalen als stabile **G**-Matrizen erweisen. Gesangsmerkmale hingegen sind männliche

Verhaltensmerkmale, die sowohl bei der Paarungserkennung als auch bei der Werbung um Weibchen verwendet werden und daher vermutlich unter sexueller Selektion stehen. Basierend auf einem speziellen Halbgeschwister-Zuchtdesign und unter Verwendung eines vergleichenden, quantitativ-genetischen Ansatzes habe ich 10 x 10 **G**-Matrizen für fünf morphologische Merkmale von drei eng verwandter Heuschreckenarten abgeschätzt und verglichen, sowie für 5 x 5 **G**-Matrizen für Gesangsmerkmale von zwei Heuschreckenarten. Dabei habe ich genetische Korrelationen zwischen den Merkmalen als Maß für genetische Kanalisierung geschätzt und geschlechtsübergreifende Korrelationen zwischen männlichen und weiblichen morphologischen Merkmalen als Maß für die Grenzen und Möglichkeiten der Evolution von Sexualdimorphismen. Außerdem habe ich Struktur der genetischen Kovarianz mit der phänotypischen Divergenz in den drei Spezies verglichen.

Im dritten Kapitel meiner Dissertation habe ich indirekte genetische Effekte von männlichen Heuschrecken auf die Reproduktionen ihrer Kopulationspartner untersucht, insbesondere vor dem Hintergrund, ob Männchen den Phänotyp der Weibchen manipulieren können, um so ihre Fitness zu maximieren. Männchen und Weibchen haben unterschiedliche evolutionäre Interessen in Bezug auf Kompromisse zwischen aktueller und zukünftiger Reproduktion, was zu einer Situation sexuell antagonistischer Koevolution führt. Ich habe fünf weibliche Fruchtbarkeitsmerkmale gemessen und die indirekten und direkten genetischen Auswirkungen auf das Männchen und das Weibchen mit Hilfe eines Halbgeschwister-Zuchtdesigns geschätzt.

Ich habe herausgefunden, dass sind die **G**-Matrizen von hochkonservierten morphologischen Merkmalen in Form und Orientierung zwischen den Arten unterscheiden. Die Ausrichtung der genetischen Variationen an der Hauptachse der Divergenz war nur bei dem jüngsten Artenpaar, *Chorthippus biguttulus* und *Gomphocerippus rufus*, ausgeprägt. Darüber hinaus trug die Divergenz in **G**-Matrizen ein phylogenetisches Signal, so dass die phylogenetisch am weitesten entfernte Art, *Pseudochorthippus parallelus*, die unähnlichste **G**-Matrix aufwies. Die Flügellänge erwies sich als das Schlüsselmerkmal, entlang dessen die **G**-Matrizen divergierten. Der Grad der genetischen Korrelation zwischen den Merkmalen innerhalb der Geschlechter war im Mittel mäßig (durchschnittlich 0,34), während die Korrelationen gleicher Merkmale zwischen den Geschlechtern groß war (durchschnittlich 0,54), insbesondere bei der Höhe der Seitenlappen des Pronotums und der Augenhöhe.

Des Weiteren fand ich heraus, dass die **G**-Matrizen der männlichen Gesangsmerkmale erhebliche Unterschiede aufweisen und sich zwischen den Arten unterscheiden. Die Gesangsmerkmale zeigen insgesamt mäßig hohe Erblichkeiten, am höchsten für Silbendauer (0,50) und Strophe (0,54) bei

Chorthippus biguttulus und Strophe (0,30) bei *Gomphocerippus rufus*. Die maximale Amplitude – ein Merkmal, das bekanntermaßen unter sexueller Selektion steht – zeigt, wie aus der Theorie vorhergesagt, eine geringe Erblichkeit (0,26). Ich fand auch eine stark negative genetische (-0,65) sowie phänotypische (-0,48) Korrelation zwischen Amplitude und Strophe-Dauer, die auf eine Amplitudenzunahme mit Abnahme der Gesangsdauer hinweist.

Im dritten Kapitel meiner Dissertation fand ich insgesamt schwache, statistisch nicht signifikante indirekte genetische Effekte von Männchen auf die Fruchtbarkeitsmerkmale ihrer Kopulationspartnerinnen. Obwohl die indirekten genetischen Effekte kurz nach der Paarung zumindest für die Größe der Eipakete, die Anzahl der Eipakete und die Anzahl der Eier pro Eipaket hoch waren, verlor sie sich der Effekt über die Zeit. Die direkten genetischen Effekte auf die Reproduktionsmerkmale waren gering bis mäßig. Die Ergebnisse deuten darauf hin, dass selbst in einem sexuellen Konfliktszenario die phänotypische Entwicklung weiblicher Reproduktionsmerkmale hauptsächlich durch direkte Selektion auf die additiv-genetische Varianz der Weibchen und nicht indirekt auf die männlichen indirekten genetischen Effekte beeinflusst wird.

In den ersten beiden Kapiteln zeigen die Ergebnisse, dass die **G**-Matrizen in Form und Orientierung sowohl für konservierte morphologische Merkmale als auch für variable Gesangsmerkmale divergiert sind. Die Differenzierung in der Morphologie erfolgte entlang der Flügellänge, und die Divergenz der **G**-Matrizen trägt ein phylogenetisches Signal. Insgesamt scheinen die evolutionären Bahnen sowohl des Gesangs als auch der Morphologie direkt von der Selektion beeinflusst zu sein

General introduction

The entire spectrum of diversity in nature has always bewildered humans perhaps from pre-historic time when our ancestors produced elaborate cave paintings inspired by the variety of animals they encountered. The more we explore, the more alluring nature gets; it elicits curiosity and makes us inquisitive about the details we observe. A closer look at the immense diversity of life and richness of species leads us to the intriguing fact of enormous variation within species. This might have sown the seeds of the revolutionary theory in Darwin's mind. Phenotypic variation is the staple for natural selection, the universal process that leads to local adaptation. Variation can arise from the underlying genetic diversity within species or from environmental variation, as phenotypes are results of the genes as well as the environment experienced (West-Eberhard, 2003). Certain phenotypes are affected by the segregation of a large number of loci, each contributing a small part to the total genetic variation. This contrasts with discrete phenotypes that fall into distinct classes and are controlled by few genes. Traits that show multilocus inheritance are known as *quantitative traits* (Falconer & Mackay, 1996). The phenotypic variance in quantitative traits can be partitioned into genetic and environmental components, which is the topic of a research field known as quantitative genetics (Lynch & Walsh, 1998). Such partitioning informs us about the genetic contribution to the phenotypic trait expression and the amount of this variation which is inherited across generations. Short term responses to selection depend strongly upon this proportion of inherited genetic variance. But it quickly gets complex when we consider correlations among traits and the complications arising from the details of the genetic architecture (Walsh, 2007). Ramifications of trait correlations and their impact on evolutionary response are the themes of this thesis.

A brief history of quantitative genetics and the concept of heritability

Quantitative genetics is the study of the pattern of inheritance of traits that are controlled by multiple loci each having a small contribution (Falconer, 1960). Understanding the genetic basis of complex traits thoroughly depends on how much the resemblance among relatives is affected by shared genes rather than environmental noise. The study of continuous traits (a short-hand expression for continuously-varying traits, in contrast to discrete phenotypes) has its roots in the late 19th century, spearheaded by Francis Galton (Galton, 1889), his disciple Karl Pearson and also W.F.R Weldon with the foundation of the biometric school of research (Provine, 1971). Galton believed in blending theory of inheritance. With the revival of Mendel's work in 1900, an intense debate started between the biometricians and the Mendelians led by William Bateson. In contrast to the Mendelian school which considered mutations with large effects as the main source of variation in discrete characters and facilitator of evolution, the biometricians perceived evolution as an outcome of selection acting on continuously varying characters (Lynch & Walsh, 1998). By plotting human

offspring height against parental height, Galton noticed that the line of best fit to the data does not align with the line of perfect inheritance (Galton, 1889). Instead, the average height of the offspring from extreme parents moved closer to the mean. Galton named this phenomenon as 'regression towards the mean' and the statistical concept of regression originates from Galton. Regression as a measure of parent-offspring resemblance is perhaps one of Galton's most important contributions to the theory of quantitative genetics and is still elemental. Galton and Pearson erected the pillars upon which modern statistics stands today and till this day, statistical concepts are of immense importance in quantitative genetics- 'the study of the continuously varying characters' (Barton & Turelli, 1989). Interpretation of Galton's regression in the light of Mendelian principles was independently done by Wilhelm Weinberg (Weinberg, 1908), Ronald Fisher (Fisher, 1919) and Sewall Wright (Wright, 1921). Fisher in his classical paper explained the theory of polygenic (multilocus) inheritance with the *infinitesimal model* which stated that phenotypes of complex traits are a product of large number of Mendelian factors with additive effects (Fisher, 1919). If we consider interaction within or between loci (dominance or epistasis), we observe that not all of the genotypic effect in parent contributes to parent-offspring resemblance. Instead a specific component of the genetic value which is the sum of the additive effects of alleles across all loci, is the factor which drives the similarity between parents and offspring and is known as the *breeding value* (Lynch & Walsh, 1998). The breeding value of an individual is the average trait value of that individual's offspring raised in an average environment given random mating. Breeding values depend on allele frequencies and is a feature of a specific individual in a specific population and environment (Lynch & Walsh, 1998).

One of the primary questions of evolutionary biology is how evolutionary processes like random genetic drift and natural selection produce evolutionary change in phenotypes. There is abundant genetic variation for most traits in nature. This variation is maintained by mutation-selection balance, overdominance, frequency dependent selection, migration, or in a few cases balancing selection (Barton & Keightley, 2002). Selection acting on phenotypes can only produce an evolutionary response when phenotypes are heritable. The phenotypic variance, i.e. the variance in phenotypic values of a trait, V_P, can be partitioned into two major components, V_G, the genetic variance and V_E, the environmental variance. The genetic variance can be further partitioned into V_A, the additive genetic variance which is the variance in breeding values, V_D, the dominance variance arising from nonadditivity of alleles within loci and the epistatic interaction variance, V_I which is the variance due to interaction between loci. Only the additive genetic variance, V_A is inherited from parents to offspring and this proportion of phenotypic variance that is heritable is estimated by the narrow sense heritability (h^2):

$$\boldsymbol{h^2 = V_A/V_P} \qquad (1)$$

Table 1: Glossary of terms used in the thesis

Terms	Definitions
Quantitative trait	A quantitative trait is a phenotype that is controlled by a large number of genes each having a small effect, and also has a continuously varying distribution in population. The expression of a quantitative phenotype is often dependent on environmental components of variation. They are also known as polygenic traits.
Infinitesimal model	A model for the inheritance of quantitative trait where an infinite number of loci with small effects are responsible for the expression of a quantitative trait. The total effect of the alleles on the phenotype is the sum of the effect of each allele.
Genetic architecture	The underlying genetic basis of traits, which can be studied at various levels. Partitioning phenotypic variance into a contribution of various terms including additive genetic variance is a way of exploring genetic architecture of traits. For multiple traits, this extends to the estimation of additive genetic variance-covariance matrix, **G**.
Genetic correlation	The covariance in additive genetic effects of two traits is known as the genetic covariance between traits. It describes the non-independent inheritance of traits and can arise due to pleiotropy or linkage disequilibrium.
Eigendecomposition	The method of factorization of a matrix into a set of eigenvalues and their corresponding eigenvectors is called eigendecomposition or eigenanalysis. In multivariate quantitative genetics, the eigendecomposition of an additive genetic variance-covariance matrix (**G**) produces eigenvectors that represents a linear combination of traits and the corresponding eigenvalue is the genetic variance associated with the eigenvector.
Animal model	A generalized linear mixed model where breeding value of each individual is fitted as a random effect. It is used in the decomposition of phenotypic variance of a trait(s) into different components.
Indirect genetic effects	The effects on the phenotype of a focal individual by the genetic architecture of an interacting individual.

It is important to understand that the estimates of additive genetic variance and dominance variance as used in quantitative genetics because it does not imply additive gene action at individual loci. Additivity in a quantitative genetic context is a function of dominance coefficients, as used in population genetics, but also of their allele frequencies. A locus with perfectly dominant gene action can still contribute to the additive genetic variance, if it contributes to parent-offspring similarity. For a diallelic locus, V_A usually peaks when the allele frequencies are equal. Hence higher estimates of V_A do not imply pure additivity as it can change with changing allele frequencies.

Fisher's theory of quantitative genetics took some time to get assimilated into evolutionary biology, but was instantly accepted by animal and plant breeders and implemented in order to improve the quality of animal and plant crops through artificially selecting different breeds (Lynch & Walsh, 1998). It was Robertson (Robertson, 1966) who connected quantitative genetics with evolutionary theory through the 'secondary theorem' which states that the selection differential *S*, is equal to the covariance between the phenotype and relative fitness.

$$\boldsymbol{S} = \boldsymbol{\sigma}[\boldsymbol{z}, \boldsymbol{w}(\boldsymbol{z})] \quad (2)$$

where *S* is the selection differential, *z* the phenotypic trait value and *w*(*z*) is the relative fitness of the phenotype *z*. This was further elaborated by George R. Price (Price, 1970) and became known as the Robertson-Price identity (Bijma, 2020, Harman, 2020). The selection differential *S* is vital in understanding evolutionary change as it is the within generational difference in phenotypic mean after selection (but before reproduction) and that of before selection (Lynch & Walsh, 1998). A change in trait means across generation is the function of heritable genetic variation and the selection differential and this is the crux of the breeder's equation.

$$\boldsymbol{R} = \boldsymbol{h^2 S} \quad (3)$$

where *R* is the response to selection, h^2 is the heritability of the trait, and *S* is the selection differential. As response to selection depends on the amount of heritable variation, no matter how strong the selection force may be, if there is no additive genetic variance for traits, i.e. when $h^2 = 0$, there will be no response. The breeder's equation as we know it today, was probably formulated by Jay Lush (Lush, 1945).

Going multivariate

The breeder's equation gives the evolutionary response to selection in the univariate case, i.e. for single traits considered in isolation. But breeders noticed quite early that selection on one trait causes a change in other traits as well (e.g. extensively discussed by Darwin, 1859). For example selecting for higher egg production decreased body weight in chicken (Dickerson, 1955). In this particular case, the cause is a negative genetic covariance between these two traits (Gyles et al., 1955). This concept of selection acting on multiple traits was integrated into evolutionary biology by Lande's pioneering paper, in which he outlines the multivariate response to selection in presence of covariation between traits (Lande, 1979).

Traits are often genetically coupled, which generates additive genetic covariance among them, the structure of which affects the direction of the response to selection (Lande, 1979, 1982, Lande &

Arnold, 1983, Blows & McGuigan, 2015). Genetic covariance between traits arises from pleiotropy where the same gene influences multiple traits, or from linkage disequilibrium which is the non-independent inheritance of alleles at two distinct loci affecting two different traits. A standardized genetic covariance is called the genetic correlation and it ranges from -1 to +1 (note that this typically refers to additive genetic covariances and correlation, but the qualifier "additive" is usually omitted). The higher the correlation in absolute terms, the more constrained is the response to selection. Hence, taking a multidimensional view on phenotypic trait space and considering the real world scenario of the existence of genetic coupling among traits, we get a palpable perspective of the intricacies of evolutionary dynamics (Lande, 1980a, Blows, 2007, Walsh & Blows, 2009). In the multivariate case, considering two or more traits, the additive genetic (co)variance between traits can be statistically summarized by the additive genetic variance-covariance matrix, **G**. The multivariate response to selection is given by the equation (Lande, 1979, 1980a):

$$\boldsymbol{\Delta z = G\beta} \qquad (4)$$

Where **Δz** is the response to selection in vector form for multiple traits, **G** is the additive genetic variance-covariance matrix and **β** is a vector of selection gradients which measures the direct force of selection on each trait. **β** is identical to the partial regression of relative fitness on a trait holding other traits constant (Lande & Arnold, 1983, Arnold, 1994). The **G** matrix is a variance-covariance matrix with variances in the diagonal and covariances in the off diagonals. In the simple bivariate case, considering additive genetic variance of trait 1 as $\mathbf{G_{11}}$ and that of trait 2 as $\mathbf{G_{22}}$ and the covariance between the two traits as $\mathbf{G_{12} = G_{21}}$, the **G** matrix can be represented as:

$$\mathbf{G} = \begin{bmatrix} \boldsymbol{G_{11}} & \boldsymbol{G_{12}} \\ \boldsymbol{G_{21}} & \boldsymbol{G_{22}} \end{bmatrix} \qquad (5)$$

Diagonalization of **G**, which involves *eigendecomposition* (a concept closely related to principal component analysis), helps us to inspect the structure and amount of genetic variation present in traits by generating a series of eigenvectors and eigenvalues. The eigenvectors are orthogonal to each other and describe the original trait space. The eigenvalues are the genetic variances associated with each eigenvector and the length of the eigenvector changes with the eigenvalues. The first eigenvector of **G** captures the maximum genetic variance and is known as the 'line of least genetic resistance to evolutionary change' or $\mathbf{g_{max}}$ (Schluter, 1996). Diagonalization gives insight into the magnitude of genetic constraint involved in response to selection by providing the number of zero eigenvalues (Blows, 2007).

The **G** matrix can be thought of as a statistical summary of a cloud of breeding values in multivariate trait space (Arnold et al., 2008). In a bivariate case if we plot the breeding values of trait 1 and trait 2 which are genetically correlated, the **G** matrix might look like the ellipse in Figure 1.

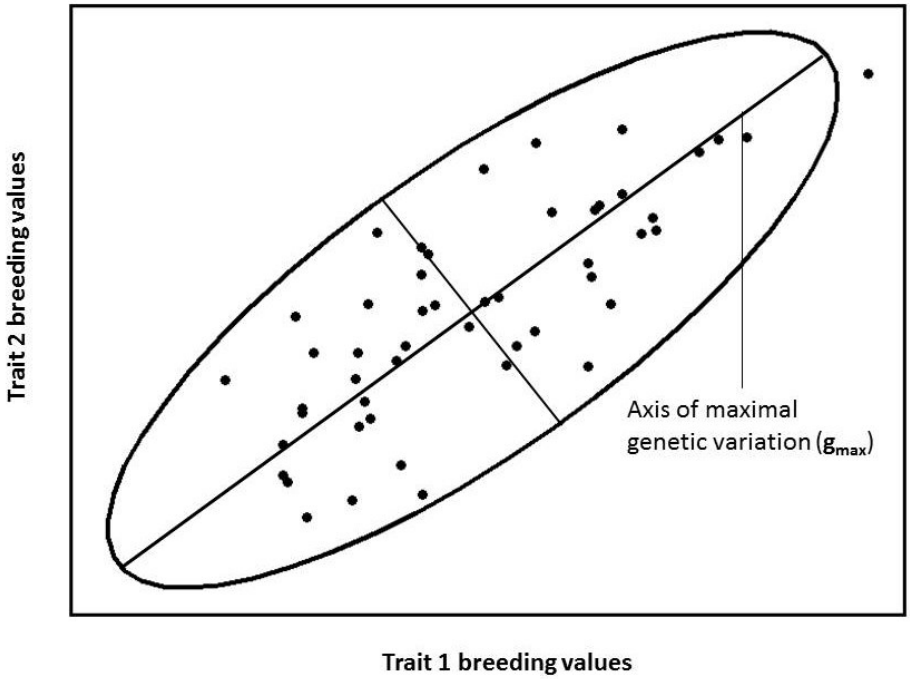

Figure 1: Individual breeding values for trait 1 and trait 2 in a hypothetical population. The ellipse shows a representation of the **G** matrix of the two correlated traits. The major axis of the ellipse gives the direction of maximum genetic variation ($\mathbf{g}_{max}$).

Evolution and stability of the G matrix

Lande (1976) merged the multivariate response to selection to the concept of Wright's adaptive landscape. The adaptive landscape describes the average relative fitness and its relationship with the average trait values. (Wright, 1932). In the case of a single trait, the adaptive landscape is a curve with a peak and for the two-trait case it is a hill (Arnold, 1992). Adaptive landscapes have two important parameters, curvature and orientation. Curvature measures how much the curve contracts in the presence of selection and the orientation determines the orientation of the hill in trait space, and is the measure of correlational selection which acts on the covariance between traits. These measures of orientation and curvature can be arranged in a matrix with the coefficients of stabilizing selection in the diagonal and that of correlational selection in the off diagonal and is known as the **γ**

matrix (Arnold, 1992, Brodie et al., 1995). Such measures of multivariate selection are of prime importance in defining the temporal dynamics of **G**.

Theoretically **G** should change between generations due to changes in allele frequencies. But Lande showed that **G** gains a lot of pleiotropic mutational inputs which cancels the effect of selection (Lande, 1980a). At equilibrium (and ignoring the effect of genetic drift) **G** will assume the shape of the multivariate adaptive landscape, and the pattern at equilibrium thus resembles the orientation of the adaptive landscape (Cheverud, 1984, Arnold, 1992). Stabilizing selection has a stronger role in shaping **G**, but in presence of substantial pleiotropic mutations, **G** can be moulded by mutations. However, if selection is strong and mutational inputs are low, **G** can change in both direction and shape. Such change can be quantified by changes in eigenvectors and eigenvalues. If selection acts on a trait combination, it will pull **G** towards the adaptive peak.

Genetic correlation creates a constraint and phenotypes cannot take a straight path to the optimum. Populations first diverge very close to $\mathbf{g}_{max}$ which is the axis of maximum genetic variation (Figure 1), and then slowly move towards the peak in a curved trajectory (Schluter, 1996). Tracing the path of **G** under directional selection is indeed a difficult problem, because the phenotypic mean changes when the population moves across the adaptive landscape changing the pattern of selection and consequently the rate and direction of evolution of **G**. These changes in **G** can also modify the direction and rate of change of the phenotypic mean (Arnold, 1992).

Predicting and estimating the stability of **G** has always been a challenge. Lande's model (Lande, 1979) has assumed the constancy of **G**. But Turelli (1988) argued that **G** remains constant under certain assumptions, such as absence of genotype-by-environment interactions, constancy of the fitness surface and the mutational covariances arising due to new mutations. Such assumptions do not always hold true under natural conditions and theoretical predictions indicate change of **G** over time. Over short evolutionary time, **G** is expected to remain similar across generations. But over long periods, **G** is predicted to diverge due to changes in covariance structure among traits and changes in the curvature and orientation of the adaptive landscape. Again, **G** can be stabilized under large population size, by correlational selection and pleiotropic mutations (Jones et al., 2003). This temporal stability of **G** is likely to be visible when compared among populations than among more distant species. The field of comparative quantitative genetics which deals with contrasting genetic architecture among populations, and also among species, can provide important insights into the issue of similarity among **G** matrices.

Stability of **G** is also an important question as the theoretical equations (for example, Equation 4) only hold true assuming **G** to be constant, but considering fluctuations, such theories cannot predict

long term response to selection. Simulation studies have shown that cigar shaped **G** matrix ellipses (elongated ellipse with a very long major axis and a small minor axis) are more stable as they constrain the evolutionary trajectory and hence slow down the pace of evolution (Jones et al., 2003). The evolutionary trajectory of **G** is thus important in understanding ancestral selection and also for exploring the underlying genetic details of population differentiation and species divergence. **G** can evolve under both selection and drift. Drift tends to cause proportional changes in **G**, i.e. changes in eigenvalues keeping the eigenvectors (orientation) relatively constant (Roff, 2000), but there is a lot of variation on this assumption (Steppan et al., 2002). Under the current theoretical quantitative genetic framework, the stability of **G** remains unpredictable, because important conditions are often unknown, and what we need is more empirical studies among populations and related taxonomic groups.

Empirical studies provide us with a realistic view of evolutionary dynamics. The above defined theories need to be tested in order to shed light on the elusive nature of stability of **G**. Some studies show that particular properties of **G** (like its orientation) can be stable between species, at least for closely related species (Roff, 2000, Begin & Roff, 2001). But if **G** evolves according to the theoretical predictions, only empirical work can resolve the ambiguities of how and when **G** changes. It is also appealing to look for the kind of traits that impart stability or cause fluctuations in **G**. Highly conserved traits like bilateral traits will be stabilized by correlational selection and pleiotropic mutations but fitness traits are extremely unstable due to strong directional selection acting on them and also because they are controlled by a large number of loci, only some of which pleiotropically affect the traits (Jones et al., 2003). Hence using such unstable traits might not be suitable for testing stability as they would naturally lead to fluctuating **G** matrices. We need more comparative studies between populations but especially between species as **G** is fundamental in predicting the evolutionary trajectory of species divergence. There is a paucity of such studies which empirically compare **G** among species and our study becomes relevant at this juncture as we test the stability of genetic architecture among species addressing the theoretical predictions. In the first chapter of my thesis, I report the work on estimation and comparison of **G** in morphological traits of three grasshopper species, two of which, *Chorthippus biguttulus* and *Gomphocerippus rufus* are rather closely related while a third species, *Pseudochorthippus parallelus*, is more distantly related.

One of the interesting outcomes of multivariate selection is sexual dimorphism. Males and females share their genome but selection on one trait in male might not favour the same in females. This gives rise to intra-locus sexual conflict which is resolved by sex specific selection ultimately giving rise to sexually dimorphic characters (Lande, 1980a, 1987). But in presence of sexually antagonistic selection, i.e. when selection acts in opposite direction in males and females, the shared genetic

variation constrains evolution of sexual dimorphism as the males and females cannot reach their specific fitness optima (Fairbairn et al., 2007). An estimate of this constraint is cross-sex genetic correlation and it is shown that sexual dimorphism increases with a decrease in cross sex correlation (Poissant et al., 2010). In the first thesis chapter, I also estimated the cross sex correlations between the male and female morphological traits as a measure of constraint to the evolution of sexual dimorphism.

Genetic architecture of male ornamental (song) traits

The second chapter of my thesis deals with the genetic architecture of male ornamental traits and its divergence across species. Spectacular male ornaments have baffled biologists for a long time, especially flamboyant traits which apparently reduce the chances of survival (Andersson, 1994). Darwin could not resolve the evolution of such characters through natural selection, and in fact this issue has been debated since Darwin introduced the concept of sexual selection (Darwin, 1871). According to Darwin, sexual selection is the differential reproduction that arises due to the competition for mates (Andersson & Iwasa, 1996). Sexual selection is the variance in mating success stemming from inter-sexual mate choice or intra-sexual competition. Though intra-sexual competition results in an advantage for the winner, the reason behind the existence and phenotypic diversity of extravagant traits in polygamous mating societies was difficult for Darwin to explain. In fact, female mate preference and its evolution has been a pressing problem in evolutionary biology.

One of the brilliant attempts to solve the riddle of showy traits was made by Fisher by the construction of a genetic framework for the evolution of female mating preference and male sexual trait in concert (Fisher, 1958, 1999). The theory of Fisherian runaway builds upon the existence of genetic correlation between male secondary sexual trait and female preference. Pleiotropic effects of genes underlying the male character and female preference is not always needed as a correlation builds up between the two as a result of assortative mating resulting from genetic variance in mating preferences, a scenario where females with extreme preference mates with males with extreme trait values (Lande, 1981). Once mating preference starts evolving, a positive feedback loop starts as the females with extreme preference selects males with extreme traits which in turn, due to the existence of the genetic correlation, select again for females with higher degree of mating preference (indirect selection). This gives way to a “runaway” process where female choice and male trait increases exponentially till counteracted by selection against the trait and the choice. This mechanism leads to phenotypic divergence between relatives and quickly paves way for speciation. Fisher’s verbal model has been extensively developed by Lande under different scenarios and Lande’s paper (Lande, 1981) laid the foundation for future works regarding the stable and unstable outcomes of runaway (Nichols & Butlin, 1989, Pomiankowski et al., 1991, Pomiankowski & Iwasa,

1993, Hall et al., 2000, Kokko et al., 2002, Ihara et al., 2003, Bailey & Moore, 2012, Greenfield et al., 2014)

Ornaments as well as preference for these ornaments are in most cases quantitative in nature and often consist of more than one trait (Lande, 1980b, Mead & Arnold, 2004). Quantitative genetics provides a sophisticated platform for studying the multivariate genetic architecture of polygenic sexually selected traits. Lande's models of sexual selection built upon Fisherian runaway are for quantitative traits and this facilitates the inclusion of other theories like "good genes", "sexual conflict", "condition-dependence" etc. into a unified quantitative genetic framework for studying the underlying genetic architecture of ornamental traits (Rowe & Houle, 1996, Mead & Arnold, 2004, Bonduriansky & Rowe, 2005, Reid, 2014). Moreover, it is important to appreciate the multivariate nature of the issue as mate preference or choice acts on a cluster of traits rather than one single trait (Prokop & Drobniak, 2016). The genetic variance covariance structure (**G**) of ornamental traits helps us to understand the quantitative genetic basis of such traits and their evolution. The response to sexual selection will also depend on the underlying genetic constraint created by genetic correlations between ornaments.

The "lek paradox" is another example where quantitative genetics play a pivotal role in understanding the architecture of sexually selected traits. The paradox is the maintenance of high genetic variation in a trait in the face of sexual (or natural) selection. The expectation is that the genetic variance gets eroded with long term selection, and yet we see sexually selected traits with high additive genetic variance (Kirkpatrick & Ryan, 1991). Several theories like genic capture, condition dependence, epigenetic resolution, mutational variance have been proposed to explain this scenario (Pomiankowski & Møller, 1995, Rowe & Houle, 1996, Bonilla et al., 2016, Dugand et al., 2019). Theories like condition dependence may work for univariate cases, but does not help resolving the paradox when multiple traits are involved (Hine et al., 2004). In spite of high heritability and strong selection if the angle between the direction of selection and the direction of genetic variance is too large or if there is no genetic variance in the direction of selection, there would be no response and the variation can be maintained (Hine et al., 2004, Van Homrigh et al., 2007).

There is a multitude of theories and models explaining sexual selection and the biology of sexually selected traits and preference (Kokko et al., 2006, Kuijper et al., 2012). But these models and theories cannot enlighten us without being empirically tested. The study of the genetic covariance structure of ornaments calls for more empirical estimation of **G** matrices. Studies involving the constancy of **G** matrices for ornamental traits are also absolutely important to the fields of evolutionary genetics and sexual selection. The assumptions of most of the models of sexual selection is a constant **G** with some exceptions (Kirkpatrick, 1996), which is different from the actual

scenario where **G** evolves due the continuous evolution of preference and ornaments. As discussed in preceding sections, the stability of **G** is a result of mutation and recombination, and selection acting on genetic variance and covariances. Whether or not **G** is stable for ornamental traits and preference is a question best answered by empirical works.

Some aspects of **G** can be stable for some characters while other aspects might evolve and lead to phenotypic divergence. Comparative quantitative genetics can give us important insights into the evolution of **G** such as under what conditions **G** changes (Steppan et al., 2002) or for what kind of traits, e.g. behavioral, morphological, ornamental and preferences traits (Prokuda & Roff, 2014). There is also a dearth of comparative studies reporting **G** for sexually selected traits. This is extremely relevant in studying the evolution of ornaments and preference as without the existence of genetic variance for these traits sexual selection cannot create an evolutionary response and even in case of the presence of genetic variance there might not be any in the direction of multivariate trait combinations of **G** resulting in no response. Though choice trials are important in assessing preference functions for male ornaments (Reinhold & Schielzeth, 2015), backing these experiments with the underlying genetic architecture of these traits is also immensely necessary.

Trait evolution and indirect genetic effects

So far we have dealt with the pattern of heritable genetic variation of traits under selection. Though Fisher (1919) partitioned phenotypic variance into genetic and environmental components, but his main focus concerned the effect of additive genetic variance on evolution (Wolf et al., 1998). However, the environment is also crucial in trait expression and can potentially alter the response to selection. Environments composed of individuals which interact with the focal individual whose phenotype is of interest, are particularly fascinating. When such a phenotype of an individual is affected by the genetic architecture of an interacting individual present in its environment, this is called an indirect genetic effect (IGE) (Wolf et al., 1998, Bijma, 2014). As the environment involves the genes of the interacting individual, it becomes heritable and hence can evolve. Phenotypes affected by the genes of an interacting individual are called ‘interacting phenotypes’ (Moore et al., 1997). IGEs are different from direct genetic effects which are the effects of inherited genes on a phenotype. In other words, IGEs exist when the phenotype of individual A is affected by the genes of an interacting conspecific B present in its environment. One of the most familiar examples of IGE are perhaps parental effects, which is the environment provided by the parents to their offspring (Kirkpatrick & Lande, 1989, Cheverud & Moore, 1994). Indirect genetic effects are likely to be present under circumstances of social interactions such as aggression, mating systems etc. but are not always recognized as such.

Presence of IGEs changes the evolutionary dynamics of quantitative traits. Conventional models of quantitative genetics are concerned with the direct genetic effects and they consider the environmental effects to be random (Wolf et al., 1998). But when the environment is heritable in presence of IGEs, it changes the response to selection as the environment also evolves. So even in the absence of direct genetic effects, there can be changes in the phenotypic mean (Wolf et al., 1998, Petfield et al., 2005, Teplitsky et al., 2010) and this is perhaps the most fascinating consequence of IGEs. Indirect genetic effects also change the genotype-phenotype relationship which is the covariance between additive genetic values and phenotypic values. Models addressing direct genetic effects consider that this relationship is accounted for by the additive genetic values (Wolf et al., 1998). But with IGEs this does not hold true, and hence the response to selection is not a function of the **G** matrix and the selection gradient alone (Arnold, 1994, Wolf et al., 1998) as part of the environment is also heritable and evolvable. This evolving environment changes the prediction of short term response to selection and can bias heritability. Hence accounting for them under particular scenarios is potentially of immense importance (Teplitsky et al., 2010).

An interesting case of IGE arises during mating and reproduction (Brommer & Rattiste, 2008). There is increased scope for IGEs because these traits are defined by interactions between individuals and the possibility of sexual conflict (Parker, 1979, Arnqvist & Rowe, 2005). This conflict over the result of male-female interactions with different fitness optima is known as inter-locus sexual conflict (Chapman et al., 2003). This conflict of evolutionary interest in males and females leads to sexually antagonistic coevolution where the two sexes evolve to produce antagonistic adaptations to reach their own fitness optima. Reproductive traits are such sets of traits where there can be possible conflict of interest between males and females especially in mating systems with multiple mating. In iteroparous species where there are trade-offs between current and future reproductions (Williams, 1966), females will tend to preserve resources for later reproduction while males will try to exhaust resources earlier to ensure and increase their relative reproductive success. Hence males may evolve strategies to manipulate female reproductive traits through indirect genetic effects to increase their own fitness. One of the potential situations where male IGEs can manipulate female fecundity traits is in iteroparous polygamous insects. Here, the female mates with multiple males and it is easy for the male to manipulate these traits through substances present in the seminal fluid or spermatophore. Seminal fluid proteins are a chief candidate for such manipulations as they can change the female physiology and stimulate the females to lay more eggs by providing extra nutrients (Wedell & Karlsson, 2003). Differential investment of resources on offspring affects the offspring fitness as they use these resources during larval growth (Qvarnström & Price, 2001). In the third chapter of my thesis, I report the study that we carried out on the effect of male IGEs on female reproductive traits on the bow-winged grasshopper, *Chorthippus biguttulus*.

Breeding designs and empirical quantification

It is possible to estimate genetic parameters from arbitrary pedigrees (Kruuk, 2004, Wilson et al., 2010). However, dedicated breeding designs greatly improve the efficiency and accuracy with which genetic parameters are estimated (Lynch & Walsh, 1998). Breeding designs are therefore at the heart of the estimation process in quantitative genetics studies. Choosing a breeding design is perhaps the single most important and pivotal step in pursuing a quantitative genetic experiment and estimating variance components. The precision of estimates of variance components and biases introduced depend on the breeding design chosen (Falconer & Mackay, 1996). Broadly speaking, there are two issues that have to be dealt with while selecting among the possible choices. Firstly, the kinds of relatives that should be used in the analysis as some kinds of phenotypic covariances between the relatives are required to estimate the desired variance components, and also in certain species certain kinds of relationships are easily available. Secondly, the experiment should be precisely designed regarding the number of individuals involved as the precision is a direct function of the total number of individuals that are measured, but the number of families involved versus the number of individuals within families has to be carefully balanced (Lynch & Walsh, 1998).

One of the breeding designs most frequently used to estimate heritability is the parent-offspring regression. It is one of the most readily identifiable relationships in most species with no influence of dominance on the parent-offspring covariance (Falconer & Mackay, 1996). Without any environmental cause of resemblance between parent and offspring, heritability is estimated as twice of the (single) parent-offspring regression slope. For a more precise estimate, both parents can be measured and the offspring phenotypes can be regressed on the mean phenotypes of the parents (mid-parent value). The slope of this midparent-offspring regression is a direct estimate of heritability (Galton, 1889). The detection of statistically significant heritability estimates from parent-offspring regression would require large sample sizes unless the heritability is high.

Another line of approach is the sibling analyses which can be of three kinds, one involving half siblings, one involving full siblings and thirdly using both of them – a full sib-half sib breeding design. Each of these designs enables the phenotypic variance to be partitioned into between family and within family components, which can again be explained as covariance between relatives (Lynch & Walsh, 1998).

Arguably, the most straightforward application of sib-ship based designs is the full sib breeding design, where *N* full sib families are analysed, each containing *n* individuals. The traditional method of estimation of the full sib variance components is the one-way analysis of variance (ANOVA). The covariance between full sibs is the variance (among family variance) among full sib families. The

major drawback of this design is that it is not possible to get a clean estimate of σ_A^2 (additive genetic variance), as the among-family variance is always inflated with the dominance variance and usually also by common environmental effect variance (resemblance among relatives due to shared environment). This comes directly from the fact that the covariance between full siblings is $\frac{\sigma_A^2}{2} + \frac{\sigma_D^2}{4} + \sigma_{E_c}^2$, where σ_A^2 is the additive genetic variance, σ_D^2 is the dominance variance and $\sigma_{E_c}^2$ is the common environmental variance. Hence the exclusive use of the full sib method should be avoided as an estimation method for heritability.

An efficient and precise way of estimating heritability and other variance components is using the nested full sib-half sib breeding design. *N* randomly selected males (sires) are each mated to ≥ 2 females (dams), so that the offspring from the same dam are all full sibs and the offspring of the sire from two different dams are half sibs (Fig.2). Traditionally the variance components are estimated from the nested analysis of variance (ANOVA). Under a paternal half sib breeding design as the one described above, four times the covariance between half sibs provide an estimate ofσ_A^2. This estimate is free from any common environmental effects, but such effects seep in once a maternal half sib breeding design (one dam mated to multiple sires) is used. As common maternal effects arise in such a design, the σ_A^2 is overestimated as four times $\sigma_{E_c}^2$ ($4\sigma_{E_c}^2$), the common environmental variance which includes the maternal effects. Thus the paternal full sib-half sib breeding design provides a very powerful platform for obtaining precise, unbiased estimates of heritability.

These sibling analyses are particularly easy for working with animals in a lab. In the quantitative genetic studies that I pursued for my thesis, all the variance components are estimated from a paternal full sib-half sib breeding design, where we set up full sib and half sib families from animals caught from the wild.

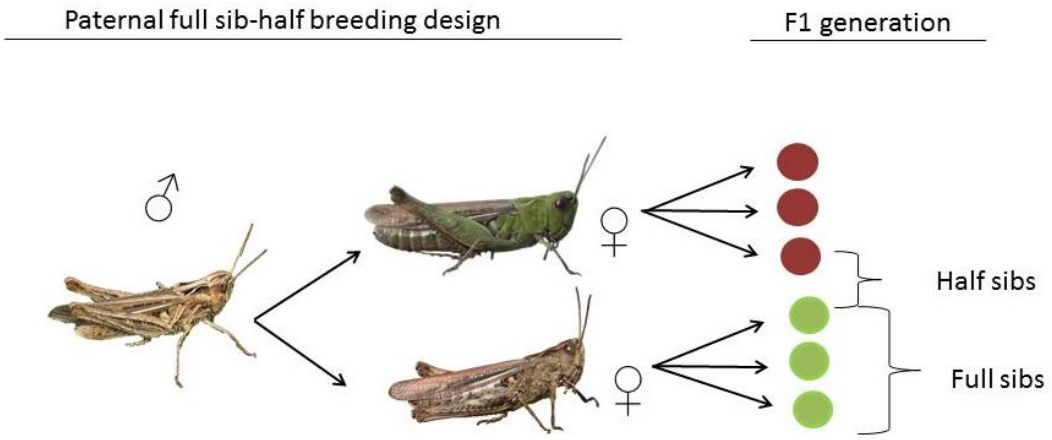

Figure 2: An example of paternal full sib-half sib breeding design using grasshoppers. The red circles and green circles from different females are sets of full sibs, whereas each red circle is a half sib to a green one. The number of full sib and half sib families involved in the study is summarized along with the total number of F1 individuals.

The animal model

An *animal model* is a form of generalized linear mixed model (GLMM) where an individual's breeding value is used as an explanatory variable for a particular trait of interest (Wilson et al., 2010, de Villemereuil et al., 2013). As was defined earlier, an individual's breeding value for a phenotypic trait is the total additive effects of its genes on that trait. The model has been used traditionally in the quantitative genetic studies of animals and plants and its structure has been well worked out (Henderson, 1950, 1975, Lynch & Walsh, 1998). Being a mixed model, it has both fixed and random effects, where fixed effects affect the mean of the distribution of the trait values. On the other hand, random effects describe factors with multiple levels, sampled from the population, and the analysis provides an estimate of the variance of the effects (Kruuk, 2004). In an animal model, a pedigree is used to estimate the additive genetic effects. For the simplest case, a single trait *y* in an individual *i* can be modelled as:

$$\boldsymbol{y_i} = \boldsymbol{\mu} + \boldsymbol{a_i} + \boldsymbol{e_i} \qquad (6)$$

where μ is the population mean, a_i is the breeding value of the individual *i,* and e_i is a term for residual error. The model is called as animal model as it is defined at the level of an individual animal (Kruuk, 2004). By fitting the breeding value as a random effect, we can estimate the variance in

breeding values which is the additive genetic variance (V_A). Besides additive genetic variance, several environmental variances and indirect genetic effects can also be estimated by fitting the right kind of random effects and pedigrees. The population pedigree is a key to such kind of an analysis as it gives an expectation of the nature of covariance of breeding values between individuals. The pedigree is generally fitted as an *n* x *n* relatedness matrix containing relatedness information between individuals. As with any mixed models, the random effects originate from a specific distribution with zero mean and unknown variance which is estimated. Hence the random effects a_i and e_i have variances of σ_A^2 and σ_R^2 respectively, and the total phenotypic variance is $\sigma_A^2 + \sigma_R^2$.

As multigenerational information from complex pedigrees can be accommodated into animal models, it is the best possible way to estimate heritabilities in wild populations (de Villemereuil et al., 2013). Using a specific breeding design is often not feasible in the wild, and also we lose out on information about other kinds of relationships if we use a particular design. Deep pedigrees which include different relationships like parent-offspring, siblings etc. and even unrelated individuals can all be fitted together in an animal model, which would have been difficult if classical linear models were used. In the wild, animals share environments and animal model can incorporated several causal components of variances like shared environment, maternal effects etc. Analyzing repeated measures data which is very common in long term studies is also possible using animal models, just like any other mixed models, by fitting an additional random effect of permanent environment. Moreover, these models allow for the analysis of unbalanced data sets. Animal models have given a huge impetus to the analysis of multivariate data and estimation of **G** matrices. As these models can be fitted to multiple trait data, genetic variances and covariances can be easily extracted.

One can either implement animal models using a restricted maximum likelihood based approach (REML) or a Bayesian approach. Both the methods have their pros and cons, for example REML based estimation is faster than Bayesian but not very ideal for analyzing non-Gaussian traits (Wilson et al., 2010). Bayesian approaches require specifications of prior distribution for unknown parameters (Gelman et al., 2013), but ideally the information should come from the data. The precision of estimates are also better in a Bayesian method especially for binary traits, and one can also fit Bayesian animal models for traits with different non-Gaussian distributions. The computation time though can be very high especially for large data sets. In my thesis, I estimated **G** matrices of morphology and songs as well as estimated indirect genetic effects using animal models with a Bayesian inference method.

G matrix comparisons

G matrices change over time, i.e. over long evolutionary time scale **G** would diverge between populations and even species due to selection and/or genetic drift. Stopping there would be underplaying the importance of exploring stability of **G** matrices. There are certain aspects of **G** which change fast, some remain stable; there are certain conditions which promote changes in **G**, and some ensure stability. Again, **G** matrices can differ in covariance structure, in orientations, in eigenstructure etc. For exploring these different facets of **G**, there are several matrix comparison methods which help to find differences among several properties of **G** (Roff et al., 2012, Aguirre et al., 2014). Here I discuss briefly four such methods which I have used in comparing **G** for my thesis, the details for each method is elaborated further in the relevant thesis chapters.

Krzanowski's subspace analysis

Orientation of genetic variances along specific multivariate axis of trait space is demonstrated by eigenvectors of **G** and the corresponding eigenvalues indicate how much genetic variance is present along the eigenvector. Hence, the first eigenvector of **G** is a linear combination of traits with maximum genetic variance for a population or species. One can define a subspace (sub-matrix) containing most of the genetic variation by including first few eigenvectors of **G**. When multiple **G** matrices are present these subspaces can be compared to test how similar they are. This can be formally analyzed by the Krzanowski's subspace analysis (Krzanowski, 1979). We can take $k/2$ eigenvectors (where k is the total number of traits) to build the subspace, the **H** matrix. Further eigendecomposition of the **H** matrix produces $k/2$ eigenvectors of **H** which contain the genetic variation of the linear trait combination shared across different populations/species. A conclusion about the difference or similarity in orientation of matrices can be reached by examining the eigenvalues of **H**.

Random skewers

Another method for testing matrix orientation is the random skewer analysis (Cheverud, 1996, Cheverud & Marroig, 2007, Calsbeek & Goodnight, 2009). This is derived directly from multivariate Lande equation, $\Delta \mathbf{z} = \mathbf{G}\boldsymbol{\beta}$ (Equation 4), where $\boldsymbol{\Delta z}$ is the change in trait means, **G** the matrix of genetic variance and covariances, $\boldsymbol{\beta}$ the linear selection gradient. Hence we get a predicted multivariate response to selection as selection vector is projected through **G** matrices. When random selection vectors (skewers) of around 1000 are projected through multiple **G**s , the responses can be compared. The angle between the response vector $\boldsymbol{\Delta z}$ and $\boldsymbol{\beta}$ gives the angle of deflection and the angle between two response vectors from different matrices measures how much the angle of deflection is when matrices are subjected to the same selection gradients.

Flury Hierarchy analysis

A way to compare the eigenstructure among matrices is the Flury hierarchy analysis (Flury, 1988). Matrices can share eigenvectors or eigenvalues, or they can share eigenvectors but no eigenvalues. A hierarchical test is built up right from the basic test of inequality among matrices, then the number of eigenvectors shared is tested, i.e. the number of common principal components (CPC) among the matrices. If the matrices share all their principal components and the eigenvalues are also equal, they are said to have satisfied all the conditions of matrix equality. There is an important philosophy behind this method that matrices can be unequal in different respects and still can share some elements of equality. Hence the blanket statements of **G** matrix stability or inequality should be explored further for deciphering aspects of **G** that might still differ or stay constant. There are various approaches for carrying out the hierarchical tests, like the "step-up" or "jump-up" approaches, and the model building approach. In my thesis, I have used the model building approach to compare matrices with Flury hierarchical analysis.

Genetic covariance tensor analysis

In the above sections, I explored how changes in direction and orientation among **G** matrices can be detected. The fourth method, genetic covariance tensor analysis (Hine et al., 2009, Aguirre et al., 2014), deals with the finer aspects of **G** matrix differentiation like determining the trait combinations for which there is a maximum change of genetic variance. In multivariate analysis, a k x k **G** matrix summarizes trait covariances for n traits. For p k x k **G** matrices, the variation among them can be described by a k x k x k x k genetic covariance tensor, $\boldsymbol{\Sigma}$. Covariance tensors are fourth order array used to describe variation in lower order variables like vectors (first order tensor) and matrices (second order tensor). Eigenanalysis of a tensor produces k (k +1)/2 or p-1 (whichever is smaller) non-zero eigenvalues and corresponding eigentensors. Eigentensors are matrices that vary in size among the **G** matrices and can further go through eigendecomposition. The leading eigentensor identifies the trait combination that has experienced the maximum change in genetic variance among the **G** matrices.

Aims of the thesis

The work described in the thesis is broadly about delving deeper into the world of multivariate quantitative genetics and exploring the underlying genetic architecture of traits. I report empirical studies on the stability of genetic architecture which is well-grounded in theory. In the first two chapters, I have worked on the estimation of genetic covariance structure of morphological and behavioral (song) traits and their subsequent comparison among three Gomphocerine grasshoppers. In the third chapter I report work on the male indirect genetic effects on female reproductive traits in grasshoppers. Specifically, the aims of the thesis are :

1. Compare the stability of **G** matrices in time using a comparative quantitative genetic approach. Specifically, I aimed to estimate and compare **G** matrices for three species of grasshoppers using a dedicated breeding design and animal model analyses.
2. Analyze the potential for genetic constraint in rather conserved morphological traits and in more variable song traits of grasshoppers.
3. Assess the cross-sex genetic correlations for conserved morphological traits in grasshoppers as an indicator of limits and potential for the evolution of sexual dimorphism.
4. Estimation of male indirect genetic effects (IGE) and female direct genetic effects on fecundity traits in a species of grasshopper where indirect genetic effects might be particularly relevant because of the potential sexual conflict over investment into current reproduction.

Manuscript overview

Manuscript I

Title: Comparative analysis of the multivariate genetic architecture of morphological traits in three species of Gomphocerine grasshoppers

Authors: Anasuya Chakrabarty, Holger Schielzeth.

Journal: Heredity, doi: 10.1038/s41437-019-0276-1 (accepted for publication: 18th September 2019).

Author contribution: Conception and experimental design: AC (30%), HS; Data analysis: AC (80%), HS; Manuscript writing: AC (70%), HS.

In this manuscript, I compared the multivariate genetic architecture of morphology across three closely related species of gomphocerine grasshoppers, *Chorthippus biguttulus*, *Gomphocerippus rufus* and *Pseudochorthippus parallelus*, the former two are more closely related than the latter one. Morphology consists of a combination of highly conserved traits which are predicted to have a more stable genetic architecture than life history or behavioural traits. It is intriguing to study whether something as conserved as morphology has diverged in this grasshopper trio, and whether such divergence follows any phylogenetic pattern. Specifically, I estimated additive genetic variance covariance matrices **G** for five morphological traits- femur length, wing length, antenna length, eye height and lobe height, and compared them using different matrix comparison methods for testing the stability of **G** matrices in these species. I also estimated between-trait genetic correlations as a measure of the degree of genetic constraint between traits, and cross-sex correlation to determine the constraint imposed by shared genetic variation on male and female traits. Finally, I compared the alignment of genetic variance with phenotypic divergence in the three species. The results demonstrated that the **G** matrices show substantial differentiation in orientation and shape and have diverged from each other. The genetic correlation between traits were low to moderate whereas the cross-sex correlations were moderate indicating scope for the evolution of sexual dimorphism, but more constrained due to shared genetic architecture for eyes and lobes. The genetic variance aligned with the main axis of divergence only for the youngest species pair whereas the genetic variance in the most distant species was oriented away from the divergence axis. Moreover, the divergence in **G** carried a phylogenetic signal, which means the **G** matrix of the most distant species was the most different. In conclusion, **G** matrices have differentiated in the three closely related species of Gomphocerince grasshoppers, even for conserved morphological traits.

Manuscript II

Title: Multivariate genetic architecture of songs in Gomphocerine grasshoppers

Authors: Anasuya Chakrabarty, Holger Schielzeth.

Accepted for publication: NA

Author contribution: Conception and experimental design: AC (30%), HS; Data analysis: AC (90%), HS; Manuscript writing: AC (90%), HS.

In this manuscript, I analyse the stability of multivariate genetic architecture of song traits in two gomphocerine grasshoppers, *Chorthippus biguttulus* and *Gomphocerippus rufus*. Songs are male ornamental traits which are produced to attract females and are plausibly under sexual selection. As they are produced by stridulatory behaviour, song parameters are phenotypically more variable than morphological traits. As sexual selection can also lead to phenotypic divergence, it is especially interesting to study the stability of genetic architecture of song traits. I estimated and compared the additive genetic variance covariance matrix **G** for five song traits, strophe duration, syllable duration, dominant frequency, onset accentuation and maximum amplitude using a half sib breeding design. I also estimated the heritabilities and genetic correlation between the traits. The entire statistical analyses consisting of the estimation of variances and covariances and correlations were based on animal models. I found that **G** matrices in the two species are different in both shape and orientation and they have diverged from each other. Traits like strophe duration and syllable duration showed high heritabilities in both species, whereas maximum amplitude, dominant frequency and onset accentuation showed lower heritabilities. Maximum amplitude has been shown to be under sexual selection and hence the low heritability justifies the theoretical prediction. I found strong negative genetic as well as phenotypic correlation between maximum amplitude and strophe duration in *Chorthippus biguttulus* which indicates that loudness increases with decreasing song duration. The genetic correlations are stronger in *C. biguttulus* compared to *G. rufus* and hence the former has a more constrained genetic architecture. The results demonstrate that the genetic architecture of male ornamental song traits has diverged and the evolutionary trajectories of these traits are most probably influenced by selection.

Manuscript III

Title: Direct and indirect genetic effects on reproductive investment in a grasshopper

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In this manuscript, I estimate male indirect genetic effects on female reproductive traits in the grasshopper *Chorthippus biguttulus* under a potential sexual conflict scenario regarding investment in current reproduction. When there is an interaction with a conspecific, then from the perspective of the focal individual, the interaction partner becomes part of the environment. If the interactive influence is heritable, then the interaction can evolve along with direct genetic effects, and such interactive influences are called indirect genetic effects (IGE). IGEs are likely to exist when males and females interact during sexual reproduction. Males and females have conflicting evolutionary interests in species with multiple mating as the females are selected to hold back resources for future investment in reproduction, whereas males are selected to increase their partner's current investment, because they have the risk of losing paternity to other males later in the season. Hence there is a trade-off between current and future reproduction which gives rise to a situation of plausible sexual conflict which might escalate in sexually antagonistic coevolution. In such a scenario, male grasshoppers have the chance to influence female fecundity traits via IGEs. I have measured five reproductive traits in females- egg length, egg pod length, number of eggs in an egg pod, number of egg pods and latency to the first egg laying and estimated male IGEs on the phenotypic variance of such traits using a half sib breeding design and animal model analyses. The results show overall low levels of male IGE on female fertility traits. IGEs were high just after mating for egg pod length, egg number and number of egg pods but slowly decreased with time. The direct genetic effects were moderate and low for number of egg pods and latency to the first egg. Hence phenotypic evolution of these fecundity traits are likely to happen by the direct selection on female additive genetic variance of the traits rather than indirectly by selection on male traits which influence female ones. Even in a relevant mating context, the effect of male IGEs turned out to be low on female reproductive traits.

Manuscript I: G matrix of morphology

Title: Comparative analysis of the multivariate genetic architecture of morphological traits in three species of Gomphocerine grasshoppers

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ARTICLE

Comparative analysis of the multivariate genetic architecture of morphological traits in three species of Gomphocerine grasshoppers

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Abstract

Evolutionary change is the change in trait values across generations, and usually occurs in multidimensional trait space rather than along isolated traits. Genetic covariation influences the magnitude and direction of evolutionary change and can be statistically summarized by the additive genetic (co)variance matrix, **G**. While **G** can affect the response to selection, it is exposed to evolutionary change by selection and genetic drift, but the magnitude and speed of these changes are poorly understood. We use comparative **G** matrix analyses to assess evolution of the shape and orientation of **G** over longer timescales in three species of Gomphocerine grasshoppers. We estimate 10×10 **G** matrices for five morphological traits expressed in both sexes. We find low-to-moderate heritabilities (average 0.36), mostly large cross-sex correlations (average 0.54) and moderate between-trait correlations (average 0.34). **G** matrices differ significantly among species with wing length contributing most to these differences. Wing length is the trait that is most divergent among species, suggesting it has been under selection during species divergence. The more distantly related species, *Pseudochorthippus parallelus*, was the most different in the shape of **G**. Projection of contemporary genetic variation into the divergence space **D** illustrates that the major axis of genetic variation in *Gomphocerippus rufus* is aligned with divergence from *Chorthippus biguttulus*, while the major axis of genetic variation in neither of the species is aligned with the divergence between *Pseudochorthippus parallelus* and the other two species. Our results demonstrate significant differences in **G** matrices with a phylogenetic signal in the differentiation.

Introduction

The efficiency and direction of adaptive evolution are not only affected by the fitness landscape, but also by the amount and structure of heritable variation (Lande 1979; Teplitsky et al. 2014). While the univariate heritability acts mainly as an efficiency filter of inheritance among generations, the structure of genetic covariance among traits also affects the direction of the response (Lande 1979, 1982; Lande and Arnold 1983; Blows and McGuigan 2015). Genetic covariance arises either from pleiotropy or from linkage disequilibrium (Lynch and Walsh 1998). Since traits are often genetically coupled by such covariation, and therefore do not evolve in isolation, only a multidimensional view of trait evolution will capture the important intricacies of evolutionary dynamics that any unidimensional analysis will miss (Lande 1980b; Blows 2007; Walsh and Blows 2009). However, despite their relevance to the efficiency and direction of adaptive evolution, we know comparatively little about how genetic covariation evolves and how temporally stable genetic correlations are in natural populations.

The additive genetic variance–covariance matrix **G** offers a statistical summary of the amount and shape of genetic variation within populations and is integral to understanding multivariate evolution in quantitative traits (Lande 1979, 1980a). Among other things, **G** can be used to predict the evolutionary response to selection. The multivariate breeder's equation $\Delta z = \mathbf{G}\boldsymbol{\beta}$ represents the predicted

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change in mean trait values $\Delta \mathbf{z}$ in multivariate trait space as the matrix product of the additive genetic variance–covariance matrix, **G** with the vector of directional selection gradients, $\boldsymbol{\beta}$ (Lande 1979). It is apparent from this relationship that selection acting on a trait will usually produce an evolutionary response on traits that are genetically correlated, even though selection does not act directly on them. Similarly, the structure of **G** can constrain adaptive evolution by reducing the efficiency of adaptive responses or modifying the direction of response to selection (Teplitsky et al. 2014). While the mathematics of this relationship is simple and well worked out, empirical advance is hampered by the difficulty in estimating multivariate inheritance as well as selection in multivariate trait space (Turelli 1988; Arnold et al. 2008).

The genetic variance–covariance structure can change by natural selection, since selection in multivariate space is not solely directional at the level of individual traits, but also correlational on trait combinations (Lande and Arnold 1983; Phillips and Arnold 1989; Sinervo and Svensson 2002). Selection on **G** can be conceptualized by the analogy of the adaptive landscape (Arnold et al. 2001). The degree of the curvature at the hilltop of the multivariate adaptive landscape is defined by the strength of stabilizing selection and the orientation of any ridge by correlational selection. Stabilizing and correlational selection can be summarized in the $\boldsymbol{\gamma}$ matrix, the matrix of nonlinear selection gradients, where the diagonal elements contain the coefficients of stabilizing or disruptive selection $\boldsymbol{\gamma}_{ii}$ and the off-diagonals of the coefficients of correlational selection $\boldsymbol{\gamma}_{ij}$ (Phillips and Arnold 1989; Blows and Brooks 2003; Blows 2007). The effects of stabilizing and correlational selection on **G** can be particularly important when the covariances arise mostly from linkage disequilibrium rather than from pleiotropy (Arnold 1992). Adaptive changes in the shape of **G** can be caused by long-term weak selection or frequent periods of strong selection, but it is difficult to differentiate empirically between these processes (Roff 2000).

As well as being shaped by selection, the genetic variance–covariance matrix may change by random genetic drift (Roff 2000; Phillips et al. 2001; Steppan et al. 2002). Drift will mostly result in proportional changes in **G** matrix, but drift can also lead to changes in shape and orientation of **G** (Phillips et al. 2001). It is difficult to disentangle the effects of drift and selection as their signatures on the structure of **G** are very similar (Merilä 1999; Phillips et al. 2001). Furthermore, it is likely that over extended periods of time both selection and drift contribute to changes in **G** and it will thus be difficult to assign changes to one cause or the other. While it would be desirable to separate the influence of selection and drift on **G** matrices, their joint effect is of relevance to how contemporary populations may respond to selection and how persistent effects of genetic covariance are in affecting evolutionary change.

The key issue in the estimation of changes in the genetic covariance matrix is the small expected effect size and the substantial sampling variance in empirical quantifications of **G**. Comparative quantitative genetic studies offer a solution to this problem, because they capitalize on the cumulative change in **G** across many generations (Schluter 1996; Steppan et al. 2002). Comparative analyses can also shed some light on the contribution of selection to changes in **G**. Divergence among species is expected to be, at least partially, caused by past selection, hence any alignment between **G** and multivariate divergence matrix **D** (where **D** represents the matrix of phenotypic differentiation estimated as the (co)variance among mean trait values across species) will suggest an effect of selection (although the effect might go both ways). Misalignment, however, is suggestive for a role of genetic drift. Comparative studies thus help to give empirical answers to the persistent conundrums about the stability of genetic covariance structure in natural populations. Surprisingly few studies have used this comparative option, probably because of the difficulty in estimating **G** in multiple species (see below).

An intriguing special case of multivariate evolution is sexually dimorphic trait expression. Sexual dimorphism is very widespread and arguably represents the most conspicuous form of intraspecific phenotypic diversity. Sexual dimorphism ultimately arises from sex-specific selection, but is typically constrained by the shared genetic variation among females and males that results in intra-locus sexual conflict if selection is sex specific (Lande 1980b; Lande 1987). Unlike functionally unrelated traits expressed in the same sex, where we may expect low genetic correlations, the ancestral genetic correlation among homologous traits in the two sexes is expected to be large and only reduced by persistent sexually antagonistic selection (Poissant et al. 2010; Griffin et al. 2013). Such cross-sex genetic correlations cannot be shaped directly by correlational selection on the same trait expressed in males and in females when the traits (in gonochorous species) are never co-expressed in any single individual and correlational selection is thus absent.

The degree of stability of **G** matrix has been an enigmatic question in the evolution of quantitative characters (Steppan et al. 2002; Arnold et al. 2008). Theory and computer simulations predict that **G** can remain stable if the shape of the fitness surface is stable and the mutational covariance is neutral with respect to **G** (Turelli. 1988; Jones et al. 2003). However, since these conditions are likely to be violated over longer evolutionary time scale, **G** is bound to change (McGuigan. 2006). Among other things, the type of traits considered affects the stability of **G**. Fitness components, for example, are predicted to have unstable **G** matrices,

while bilateral traits have a more stable structure of **G** due to high correlations between mutational effects and strong correlational selection on both sides (Jones et al. 2003). However, there is no general theoretical answer to the stability of **G** in natural populations, since factors and conditions that can stabilize or destabilize **G** are likely to coexist.

Empirical studies allow insights into the evolutionary dynamics of **G**. Previous studies report stable **G** among populations of the individual species for life history (Spitze et al. 1991; Delahaie et al. 2017), morphology (Brodie 1993; Delahaie et al. 2017), and behavior (Brodie 1993). In other cases, **G** matrices for morphology, life history, and behavior seem to differ between populations (Shaw et al. 1995; Doroszuk et al. 2008; Careau et al. 2015; Karlsson Green et al. 2016; Sniegula et al. 2018) or are differentiated by habitat or ecotype (Calsbeek et al. 2011; Eroukhmanoff and Svensson 2011). Similarly, longitudinal studies on single populations of passerine birds have reported either temporal stability for reproductive traits (Garant et al. 2008) or remarkable changes as for morphological traits (Björklund et al. 2013). Furthermore, experimental populations of inbred lines in *Drosophila* show divergence for morphological **G** matrices (Phillips et al. 2001). Between-species comparisons that build on longer divergence times are rather few. As for within-species comparisons, there is evidence for stability of **G** matrices (Bégin and Roff 2003) as well as evidence for **G** matrix divergence for morphology (Paulsen 1996; Roff and Mousseau 1999; McGlothlin et al. 2018) in among-species comparisons. Differences among studies might reflect differences in traits, ecological context, population history, etc. Overall, there seems to be substantial variation in outcomes, a little more evidence for **G** matrix divergence on longer timescales, but also some cases of rather rapid changes.

We measured five morphological traits and performed an among-species comparison to test empirically for **G** matrix stability in a group of grasshoppers. The traits measured are femur length, wing length, antenna length, eye height, and lobe height, separately in both the sexes. Specifically, we studied three species of grasshoppers from the subfamily Gomphocerinae, a clade of grasshoppers with about 230 species, world-wide distribution (Cigliano et al. 2017) and with rather conserved general morphology. Though exact divergence times are unknown, the Gomphocerinae have started to diversify about 30 Mya (Contreras and Chapco 2006). Within this clade we selected two closely related species, *Chorthippus biguttulus* and *Gomphocerippus rufus*, that show about 4.8% mitochondrial sequence divergence (Contreras and Chapco 2006). For comparison, we chose a more distantly related species, *Pseudochorthippus parallelus* (Nattier et al. 2011; Vedenina and Mugue 2011). We predict the genetic (co)variance structure to be more similar between the two more closely related species as compared with the more diverged species. Furthermore, we predict an alignment between **G** and the divergence matrix **D** if either selection has acted to align **G** with the vector of selection or if **G** has constrained the response to selection to be aligned with the main axis of genetic variation. We expect less alignment if drift has contributed substantially to **G** matrix divergence.

We performed a full matrix comparison among the three species using various matrix comparison tools (Roff et al. 2012). Comparisons advanced in five steps of increasing complexity. (1) We estimated heritabilities, shared environmental effects, and permanent environment for all five morphological traits in both males and females, and compared the contribution of sources of variances to phenotypic variation among species and sexes. (2) We compared the multivariate trait covariation among five morphological traits and compared them among the sexes and among species. (3) We compared between sex correlations within species to address the constraint to evolution of sexual dimorphism. (4) We used four different matrix comparison methods to assess the overall (dis)similarity in the structure of genetic (co)variation and how this similarity depends on phylogenetic relatedness. (5) We assessed whether the overall genetic variance–covariance structure is aligned with the phenotypic divergence among the three species. Our work contributes to the pool of empirical evidence of **G** matrix divergence over longer time scale through among-species comparison of morphological quantitative traits.

Materials and methods

Study organisms

We studied three species of Acridid grasshoppers, *Chorthippus biguttulus*, *Pseudochorthippus parallelus*, and *Gomphocerippus rufus* (for brevity we refer to them without the genus name in the following). All of the three species are sexually dimorphic in morphology: females are generally bigger than males, but wings are relatively shorter in females than in males and antennae show significant sexual dimorphism (Table S1). We collected *biguttulus* and *parallelus* from in and around Bielefeld, Germany (52°01′ N; 08°28′ E). These two species prefer different habitats: *biguttulus* inhabits dry grasslands, whereas *parallelus* is found in lush green meadows. However, both species have wide ecological amplitude and co-occur in many places. The third species *rufus* was collected from Tübingen, Germany (48°30′ N; 09°04′ E), where it occurs on semi-open slopes with tall grasses and herbs. Only final (fourth) instar nymphae were caught from the field to ensure virginity. Nymphae were kept in netted plastic cages and provided with grass as a food source. Upon emergence as adults,

virgin males and females were kept separately in large mesh cages ($47.5 \times 47.5 \times 90\ cm^3$).

Breeding design

We set up a paternal half-sib breeding design in the laboratory at Bielefeld University. Each male was mated to two virgin females to form paternal half-sib families. Females were housed and paired separately in mesh cages ($22 \times 16 \times 16\ cm^3$). Males were swapped between their assigned female cages every 2–3 days until the male died. Females that died were replaced by new virgin females if the male was still alive. Each cage was provided with sand pots as egg-laying substrate. The sand was sieved once per week for egg pods (ca. 1 cm long solid structures containing typically 6–12 eggs, and only occasionally more (Chakrabarty et al. 2019)). Egg pods were then collected and kept in petri dishes lined with filter paper. Each pod was kept on a separate dish and dishes were sprayed regularly to keep the eggs moist. Egg dishes were kept in refrigerators at 0–10 °C for a period of at least 3 months starting from October.

F1 animals

Petri dishes with egg pods were taken out of the refrigerators in five cohorts between early January and early June. Upon hatching, individuals from the same egg pod were kept in the same mesh cage (dimensions: $22 \times 16 \times 16\ cm^3$) and were provided with ad libitum food (freshly cut grass provided in a vial with water). Egg pods produced on average 6 hatchlings per pod and the mean and SD of surviving hatchlings per egg pod were: 3.21 ± 2.28 in *biguttulus*, 4.13 ± 2.35 in *rufus*, and 3.13 ± 2.10 in *parallelus*. Hatchlings were kept at a temperature of 25–30 °C and 35–55% relative humidity. Newly emerged adults were individually marked with numbered bee tags and transferred to larger communal cages (dimensions $43.5 \times 43.5 \times 93\ cm^3$) in groups of ~25 individuals. The number of F1 animals that survived to adulthood were 1237 *biguttulus*, 897 *rufus*, and 390 *parallelus* that hatched from 383 egg pods in *biguttulus*, 217 in *rufus*, and 120 in *parallelus*. The total number of full-sib families was 112 in *biguttulus*, 70 in *rufus*, and 66 *parallelus*. The numbers of half-sib families were: 73 for *biguttulus*, 48 *rufus*, and 51 in *parallelus*. The number of males in *biguttulus* is 656 and number of females is 581, in *rufus* is 463 and 434, and in *parallelus* is 195 and 195.

Morphometrics

Standardized photographs of both males and females (adults only) from F1 generation were taken after collection or natural death. Hind legs, forewings (tegmina), and antennae were first detached from the main body. Photographs of the antennae, forewings, and hind legs were taken on a white background with a scale next to the body parts. For pictures of pronotal lobes and eyes, the animal was placed on a dish full of fine-grained sand with a scale next to it. The sand allowed adjusting the body for plain dorsal and lateral views. An artificial light source was used for the photographs, which were all taken by a Fuji camera (FinePix HS35 EXR). Pictures were analyzed using the software ImageJ 1.46r (Schneider et al. 2012). We measured post-femur length, forewing length, length of the antennae, height of the pronotal lobes, and the vertical diameter of the eye. We refer to those as femur length, wing length, antenna length, lobe height, and eye height in the following. All traits were sexually dimorphic with weakest sexual dimorphism in eye height (Table S1). Each measurement was calibrated using a 20 mm scale.

Individuals of two species *rufus* and *biguttulus* are all long-winged, while the wings are short in *parallelus*. Specifically, female *parallelus* shows forewings about half the length of the abdomen and hindwings that are reduced to stubs. Male *parallelus* also shows very narrow, short hindwings, but slightly longer forewings that cover most of the abdomen. Both sexes of *parallelus* are incapable of flight, which would require developed hindwings. However, most natural populations harbor individuals with fully developed fore- and hindwings at low frequencies, and this wing length polymorphism is apparently environmentally induced (Harrison 1980; Ritchie et al. 1987). These individuals with fully developed wings are called macropterous. While all our founding individuals were short-winged, we found 13 macropterous individuals (1.8%) from seven different half-sib families among the offspring. Because these individuals represented outliers with respect to the normal distribution of wing length in the population (Fig. S1), we excluded the wing lengths of these individuals from the analysis of this trait. We also performed an analysis that excluded macropterous individuals entirely, but this hardly affected the results (Table S5).

Statistical analyses

All models were fitted in R 3.5.1 (R Core Team 2018) under a Bayesian framework as implemented in the MCMCglmm package (Hadfield 2010). We fitted multivariate animal models and extracted the posterior distribution of **G** as well as other (co)variances. For each individual trait the key components to the model were

$$\mathbf{y} = \mathbf{X}\boldsymbol{\beta} + \mathbf{Z}_1\mathbf{a} + \mathbf{Z}_2\mathbf{s} + \mathbf{Z}_3\mathbf{i} + \mathbf{e} \quad (1)$$

where $\mathbf{y}$ is a vector of trait values, $\boldsymbol{\beta}$ is the vector of fixed effects, $\mathbf{a}$ is the vector of additive genetic effects, $\mathbf{s}$ is the vector of shared environmental effects, $\mathbf{i}$ is the vector of

individual identity effects (individuals have two measurements for each bilateral traits), and **e** is the vector of residual errors. The shared environmental effect is the part of the phenotypic variance originating from individuals sharing the same egg pod (hence also the same mother) and shared rearing conditions in the same nymphal cage. The individual identity effect is the part of the total variance that is reproducible between sides within individuals (hence excluding most measurement error and fluctuating asymmetry) beyond similarity among siblings. The residual component consists of measurement error and fluctuating asymmetry between the two sides. $\mathbf{Z}_1$, $\mathbf{Z}_2$, and $\mathbf{Z}_3$ are the respective incidence matrices for three vectors of random effects (based on pedigree relationships, egg pod identities, and individual identities, respectively) and $\mathbf{X}$ is the design matrix for the fixed effects. Heritabilities were calculated with the residual component removed, since we were not interested in fluctuating asymmetry and the individual repeatable component is deprived of measurement error and thus closer to the genetic signal.

We fitted ten-trait models (five morphological traits expressed in both sexes) for all species in order to estimate 10×10 **G** matrices (and we will refer to the dimension of **G** as n in the following). Side was fitted as a fixed effect with two levels (coded as numeric, left $= -0.5$ and right $= 0.5$). For the estimation of individual identity effects, we split the 10×10 matrix into two 5×5 matrices for males and females, because within-individual covariation cannot exist between the sexes. By doing so, individual-specific covariances between the sexes were effectively constrained to zero. We estimated the between sex correlations as well as the within sex between trait correlations from the above models.

We used parameter expanded (half Cauchy) priors for our model as they are less informative than regularly used inverse-Wishart priors (Gelman 2006). The reason for using a weakly informative prior is to ensure that the information comes chiefly from the data. The degree of belief parameter ν was set to $\nu = 11$ for all the random effects except for the individual identity. We also fitted models with $\nu = 9$, $\nu = 10$, and $\nu = 12$ for sensitivity assessment, but the choice of ν had rather little influence on critical measurements (see Figs. S5–S7). For individual identity, the 10×10 matrix was split into two 5×5 matrices, one for males and one for females with $\nu = 6$ for each matrix. The ν for residual effects was set to 0.002. The posterior distribution of each model was estimated from 1,100,000 MCMC iterations with a thinning interval of 1000 and a burn-in period of 100,000. We ran two independent chains per model yielding a total of 2×1000 samples form the posterior distribution for each parameter. Model convergence was visually inspected using the trace plots and using Gelman and Rubin diagnostics (Gelman and Rubin 1992).

Matrix comparisons

We used four established matrix comparison methods that allow exploring different aspects of variance–covariance matrices with subtly different inferences. The Krzanowski's subspace analysis determines whether the subspaces containing maximum genetic variation are similar across species. The random skewer analysis allows evaluating differences in orientation of **G** matrices. The Flury hierarchy analysis implements a formal assessment of the number of shared eigenvectors. The tensor analysis quantifies specifically the differences among variances and covariances across **G** matrices.

We compared raw matrices on the original scale, because trait units were identical and size difference were not excessive. However, in order to remove size effects, we also compared matrices after division by the square of trait means (Houle 1992) to account for allometric scaling with size. Results for matrix comparisons on mean-standardized variance–covariances matrices were qualitatively similar to unstandardized matrices.

Krzanowski's common subspace analysis

One approach to compare genetic architecture is to identify the part of **G** containing most of the genetic variance and to test whether this part overlaps among species. Krzanowski's subspace analysis is a method to evaluate which part of **G** contains the maximum variance and whether the eigenvectors explaining most of the genetic variance is similar across species/populations (Krzanowski 1979; Aguirre et al. 2014; Gosden and Chenoweth 2014). The similarity among subspaces of **G** matrices that captures the greatest amount of genetic variance can be tested using this analysis. The common subspace **H** among the $p = 3$ species is given by (Krzanowski 1979)

$$\mathbf{H} = \sum_{t=1}^{p} \mathbf{A}_t \mathbf{A}_t^T \tag{2}$$

where $t = 1, \ldots, p$ indexes species and $\mathbf{A}_t$ contains the subset of k_t eigenvectors of $\mathbf{G}_t$ as columns and k is an integer smaller than n (the dimensions of **G**) that is chosen a priori (see below). These k vectors define the dominant subspace of the three **G** matrices and only a k dimensional comparison is interpretable as adding any further dimension will make that orthogonal to one of the species subspaces ($k = \min(k_i), = 1, \ldots, p$) (Krzanowski 1979). We chose to analyze the common subspace using a fixed number of $k = 5$ eigenvectors of **G** following previous studies that used k equal to half the size of the original **G** matrix (Aguirre et al. 2014; Gosden and Chenoweth 2014).

We conducted an eigendecomposition of the **H** matrix, where the five eigenvectors $\mathbf{h}_i$ of **H** contain the genetic variation of the linear trait combination that is shared across the three species. The eigenvalues of **H** can reach a maximum value of $p = 3$. At the limit, an eigenvector with an associated eigenvalue of 3 shows that a linear combination of traits completely explains the genetic variance that is shared across the three species (Gosden and Chenoweth 2014). A departure of an eigenvalue from this maximum value of $p = 3$ shows that the trait combination of the corresponding eigenvector of **H** cannot be rebuilt from the $k = 5$ eigenvectors of **G** in at least one of the species. This indicates that the dominant part of the **G** matrix of at least one species is not perfectly aligned with the eigenvector of **H** (Aguirre et al. 2014; Gosden and Chenoweth 2014).

The difference in alignment can be measured by the angle $\boldsymbol{\delta}_t$ between each eigenvector of **H** and the subspaces of species t (Krzanowski 1979; Gosden and Chenoweth 2014)

$$\boldsymbol{\delta}_t = \cos^{-1}\left\{\sqrt{\mathbf{h}_i^{\mathrm{T}}\mathbf{A}_t\mathbf{A}_t^{\mathrm{T}}\mathbf{h}_i}\right\} \tag{3}$$

This comparison when done under a Bayesian framework can use the samples form the posterior distributions of **G** matrices, and thus gives a measure of uncertainty in estimates (Aguirre et al. 2014). In order to test statistically whether the observed differences among the **G** matrices of the three species are caused by sampling variance, we compared the observed data from the subspace analysis with a null model where we expect the **G** matrix differences are solely due to random sampling. Randomized **G** matrices were created from the posterior predictive breeding values of the observed set of **G** matrices followed by an estimation of the covariance of breeding values among traits. p values were calculated as the proportion of randomized samples that show equal or smaller values than the original MCMC samples. The R code for the analysis was adapted from Aguirre et al. (2014).

Random skewers analysis

Random skewer analysis is a method for comparing the population-level consequences of matrix differences (mostly in shape) when a population is exposed to random linear selection gradients (Cheverud 1996; Cheverud and Marroig 2007; Roff et al. 2012; Aguirre et al. 2014). This method primarily compares differences in matrix orientations. Random skewer analysis builds on the multivariate breeder's equation, $\Delta\mathbf{z} = \mathbf{G}\boldsymbol{\beta}$, where $\Delta\mathbf{z}$ is the vector of trait changes and $\boldsymbol{\beta}$ is a vector of selection gradients, and makes use of its biological interpretation. Randomly generated selection vectors $\boldsymbol{\beta}$ are projected through the **G** matrices, and the predicted response to selection, $\Delta\mathbf{z}$, is calculated. The angle θ between $\Delta\mathbf{z}$ with $\boldsymbol{\beta}$ quantifies the amount of deflection and the angle between two $\Delta\mathbf{z}$ from different matrices measures the difference in the direction of deflection when exposed to the same vector of random selection gradients.

We generated 1000 random selection vectors sampled from a multivariate normal distribution with uncorrelated axes. All 1000 random skewers were projected through MCMC samples of each **G** matrix generating a posterior distribution of response vectors (applying the same 1000 skewers to the two chains). The angle between response vectors was calculated as

$$\boldsymbol{\theta} = \cos^{1}\frac{\mathbf{v}_1^{\mathrm{T}}\mathbf{v}_2}{\sqrt{\mathbf{v}_1^{\mathrm{T}}\mathbf{v}_1.\mathbf{v}_2^{\mathrm{T}}\mathbf{v}_2}} \tag{4}$$

where $\mathbf{v}_1$ and $\mathbf{v}_2$ are the two vectors to be compared. Since angle calculations were done on each MCMC sample, we extracted 2000 angles that together represent the posterior distribution of the estimate.

Flury hierarchy analysis

Flury hierarchy is an approach of matrix comparison where a series of models are built and ranked, starting from matrix inequality to equality (Flury 1988; Arnold and Phillips 1999; Phillips and Arnold 1999; Steppan et al. 2002; Roff et al. 2012). It is a test of overall similarity of matrices through a series of hierarchical tests. One of the most important contributions of Flury hierarchy in comparing **G** matrices is the stepwise analysis of matrix similarities and differences. As matrix comparison is a multivariate exercise, there are several possible states between the extreme conditions of matrix equality and inequality (Arnold and Phillips 1999). Besides this broad classification of equal or unequal, matrices can differ in other characters like difference in eigenvalue or eigenvector. If the matrices have similar eigenvectors but their eigenvalues differ by a constant, then the matrices are said to be proportional. Matrices can also differ in having different eigenvalues but having all eigenvectors in common. This is tested by the common principal component model (CPC) that assumes eigenvectors to be identical. Sharing of eigenvectors can also be partial, which is tested by partial CPC models (Phillips and Arnold 1999) that allow for 1 to $n-2$ eigenvectors to be identical between matrices, where n is the dimension of the **G** matrices.

An approach for comparing the model fit by Akaike information criteria (AIC) was described by Flury (Flury 1988; Phillips and Arnold 1999). AIC adjusts the log-likelihood of a particular model for the number of parameters used to fit a particular model. Models with smaller AIC values are considered better fits. We used the R

package 'cpc' (Pepler 2015) to perform the Flury hierarchical tests using the model building approach, on the three species-specific **G** matrices. We reiterated tests for our 2000 MCMC samples to get a posterior distribution of AIC values and ranked models by average AIC.

Genetic covariance tensor analysis

Genetic covariance tensor analysis is a method to explore and determine directions in which divergence in **G** matrices occur among populations or species (Hine et al. 2009; Aguirre et al. 2014). It quantifies the variance in (co)variances across **G** matrices and thus offers a quantitative summary of matrix differences. The structure of those variances in (co)variances can then be analyzed by eigenanalyses.

In multilinear algebra, covariance tensors are fourth order arrays that are used to define variation in lower order variables like vectors (first order tensors) and matrices (second order tensors). The covariance structure of a set of traits of a single species as summarized by a two-dimensional **G** matrix thus represents a second order tensor with elements indexed by i and j for the 1 to n traits. If more than one species are concerned, then the covariance elements of **G** can be characterized by a four-dimensional genetic covariance tensor $\boldsymbol{\Sigma}$, which is indexed by i, j, k, and l each varying from 1 to n traits in two or more **G** matrices (traits indexed i and j in one matrix and k and l in the other). Elements of $\boldsymbol{\Sigma}$ are thus defined by the covariance in (co) variances of multiple **G** matrices as (Hine et al. 2009)

$$\sum_{ij,kl} = \boldsymbol{cov}\left(\boldsymbol{G}_{ij}, \boldsymbol{G}_{kl}\right) \tag{5}$$

The genetic covariance tensor $\boldsymbol{\Sigma}$ can also be summarized by a symmetric matrix **S** with dimensions $m \times m$, where $m = \frac{n(n+1)}{2}$. The **S** matrix summarizes $\boldsymbol{\Sigma}$ in 2D format (see Hine et al. 2009 for details).

An eigenanalysis of $\boldsymbol{\Sigma}$ can be done in a similar fashion like an eigenanalysis of a **G** matrix, except that what represents an eigenvector in the case of an eigendecomposition of a two-dimensional **G** is represented by a two-dimensional eigentensor (a matrix, symbolized by **E**) in the case of eigendecomposition of a four-dimensional $\boldsymbol{\Sigma}$. As in eigenanalysis of **G**, each eigentensor **E** is associated with an eigenvalue that quantifies how much variation among **G** matrices is captured by each **E** (Hine et al. 2009; Careau et al. 2015). The maximum number of nonzero eigenvalues of $\boldsymbol{\Sigma}$ is $\frac{n(n+1)}{2}$ or $(p-1)$, whichever is smaller, where n is the dimensions of the **G** matrices and p the number of **G** matrices to be compared (Hine et al. 2009; Aguirre et al. 2014). In our study, this number is $p - 1 = 2$. Further exploration of the variation among **G** matrices can be done using orthogonal linear combination of traits, which portray the independent changes among **G** matrices, by eigenanalysis of the eigentensor **E** where the eigenvectors of **E** are denoted as **e**. If the largest eigenvalue of an **E** is close to 1, then the change in covariance pattern as defined by the eigentensor can be attributed to the change in V_A for a particular trait combination.

We used a Bayesian framework, as outlined in Aguirre et al. (2014), to determine which independent facets of the genetic covariance structure as described by the tensor show significant variation among three species of grasshoppers. We determined $\mathbf{S}_i$, the matrix representation of a tensor for the ith MCMC sample of the set of three **G** matrices and extracted the posterior mean of **S** based on all 2000 MCMC samples and estimated the variance among **G** matrices α_{ij}, explained by each eigenvector. This enabled us to calculate the amount of additive genetic variation V_A in the direction of greatest genetic variation among the three species-specific **G** matrices (Careau et al. 2015). The posterior distribution of α_j contains the uncertainty in the variance of the covariance structure as described by each eigentensor **E**. This posterior distribution of α_j is then compared with the posterior distribution extracted from the null model, where the variation among matrices is solely caused by sampling variation after randomizing breeding values.

Alignment of G with D

We quantified phenotypic divergence in morphospace among the species as a proxy for the long-term direction of evolutionary change in the past (Schluter 1996). We constructed a species mean trait value variance–covariance matrix **D** across all three species and the ten sex-specific traits. Since subjects were raised in the same environment, phenotypic means are expected to be representative for genetic divergences among species. We used eigenanalysis of the **D** matrix to quantify the first and second principal axes of species divergence. This can be done separately by sex (with five traits in each sex) and for sexes pooled (ten traits, five in each sex). Both are of interest because the first one captures sex-specific divergence and the second captures species-specific divergence. Breeding values estimated from our MCMCglmm animal model were then projected into divergence space for display.

Results

Heritabilities

We estimated heritabilities and other variance components (Fig. 1) for ten sex-specific traits from multivariate animal models. Heritabilities averaged $h^2 = 0.36$ (Table 1). Females tended to show lower heritabilities than males

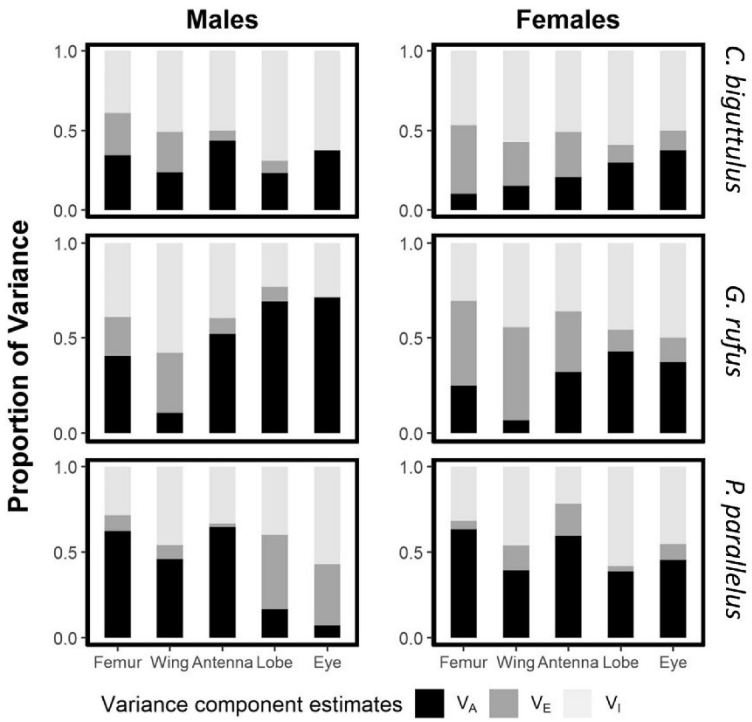

Fig. 1 Sex-specific components of phenotypic variance across five morphological traits in *Chorthippus biguttulus*, *Gomphocerippus rufus*, and *Pseudochorthippus parallelus*. V_A is the additive genetic variance; V_E shared environmental variance, and V_I the individual identity variance

(0.32 in females vs. 0.39 in males). Though the heritabilities of the sexes overlapped on a trait-by-trait basis, we find the same tendency replicated over most traits and species (Table 1).

Genetic correlations

Genetic correlations between traits within sexes were moderate (average $r_G = 0.34$) with strongest correlations in *parallelus* (average $r_G = 0.45$) and lowest in *rufus* (average $r_G = 0.23$, Table 2). Correlations tended to be higher in males (average $r_G = 0.38$) than in females (average $r_G = 0.30$) and in traits that involved femurs, lobes or eyes ($r_G = 0.35$–0.43) than correlations that involved wings or antennae ($r_G = 0.24$).

We estimated cross-sex genetic correlations r_{MF} between males and females in all three species. Cross-sex correlations were moderate and positive (average $r_{MF} = 0.54$, Table 2), were similar across species (lowest in *parallelus*, average $r_{MF} = 0.48$, highest in *biguttulus*, average $r_{MF} = 0.58$) and higher for femur, lobes, and eyes ($r_{MF} = 0.58$–0.66) than for wings and antennae ($r_{MF} = 0.40$–0.44). Among-trait correlations across sexes were weaker (average $r_G = 0.27$) than correlations within traits across sexes and across-traits within sexes, with correlations involving femur, lobes, or eyes being higher ($r_G = 0.28$–0.36) than correlations involving wings or antennae ($r_G = 0.16$–0.18) (Table S3).

Subspace analysis

We used the first five eigenvectors of **G** (together explaining 97% of the variance in **G** in *biguttulus* and *parallelus* and 96% in *rufus*) to test whether the dominant subspaces **A** of **G** were shared among the three different species. Eigenvalues were compared with a value of 3 that would indicate identical subspaces. In order to accommodate sampling variance, we compared estimated with randomized eigenvalues. The first four eigenvalues of **H** were significantly lower than 3, which indicate significant differences in orientation among the **G** matrices of the three species (Fig. 2). The angle between each eigenvector of **H** and each of the k subspaces of **G** shows only minor differences among species (Table S8). If the angles are close to zero, the specific eigenvector of h is closer to the species subspace and explains the genetic variance better for that subspace. The overlap of the credibility intervals for the angles shows that there is little difference in degree of divergence among the subspaces, though the subspaces themselves have diverged in orientation.

Random skewer analysis

The random skewer analysis showed marked difference of genetic covariance matrices across three species. Based on 2000 random skewer projections, the angle of deflection in

Table 1 Sex-specific additive genetic variances and heritabilities (±SE and 95% CI) of five morphological traits in *Chorthippus biguttulus*, *Gomphocerippus rufus*, and *Pseudochorthippus parallelus*

Species	Sex	Femur	Wing	Antenna	Lobe	Eye
Additive genetic variance						
C. biguttulus	Male	0.078 ± 0.047 (0.019–0.222)	0.137 ± 0.096 (0.022–0.427)	0.175 ± 0.053 (0.086–0.297)	0.006 ± 0.004 (0.001–0.019)	0.003 ± 0.001 (0.001–0.007)
	Female	0.048 ± 0.072 (0.000–0.299)	0.145 ± 0.091 (0.012–0.372)	0.075 ± 0.037 (0.019–0.160)	0.008 ± 0.008 (0.001–0.035)	0.003 ± 0.002 (0.001–0.010)
G. rufus	Male	0.080 ± 0.035 (0.025–0.162)	0.060 ± 0.068 (0.000–0.255)	0.275 ± 0.111 (0.108–0.542)	0.009 ± 0.003 (0.004–0.015)	0.005 ± 0.001 (0.003–0.008)
	Female	0.159 ± 0.093 (0.041–0.449)	0.076 ± 0.100 (0.000–0.375)	0.180 ± 0.090 (0.048–0.395)	0.015 ± 0.008 (0.005–0.039)	0.003 ± 0.002 (0.001–0.009)
P. parallelus	Male	0.142 ± 0.060 (0.039–0.260)	0.364 ± 0.181 (0.077–0.787)	0.407 ± 0.161 (0.120–0.728)	0.005 ± 0.005 (0.000–0.017)	0.001 ± 0.001 (0.000–0.004)
	Female	0.351 ± 0.188 (0.042–0.689)	0.385 ± 0.252 (0.011–0.957)	0.175 ± 0.085 (0.012–0.333)	0.012 ± 0.010 (0.000–0.032)	0.005 ± 0.003 (0.000–0.011)
Heritability						
C. biguttulus	Male	0.335 ± 0.170 (0.087–0.790)	0.232 ± 0.141 (0.040–0.615)	0.433 ± 0.112 (0.224–0.662)	0.230 ± 0.133 (0.032–0.594)	0.355 ± 0.123 (0.158–0.665)
	Female	0.097 ± 0.132 (0.000–0.546)	0.149 ± 0.088 (0.013–0.363)	0.205 ± 0.094 (0.053–0.413)	0.279 ± 0.206 (0.053–0.937)	0.348 ± 0.181 (0.132–0.911)
G. rufus	Male	0.396 ± 0.147 (0.138–0.714)	0.103 ± 0.111 (0.000–0.427)	0.511 ± 0.165 (0.224–0.869)	0.678 ± 0.149 (0.364–0.947)	0.705 ± 0.140 (0.408–0.954)
	Female	0.245 ± 0.128 (0.069–0.621)	0.066 ± 0.084 (0.000–0.315)	0.316 ± 0.138 (0.091–0.625)	0.423 ± 0.178 (0.159–0.919)	0.365 ± 0.182 (0.101–0.857)
P. parallelus	Male	0.610 ± 0.210 (0.185–0.954)	0.450 ± 0.192 (0.107–0.848)	0.635 ± 0.201 (0.222–0.967)	0.167 ± 0.136 (0.001–0.497)	0.065 ± 0.078 (0.000–0.275)
	Female	0.610 ± 0.272 (0.088–0.985)	0.379 ± 0.218 (0.012–0.809)	0.581 ± 0.248 (0.042–0.955)	0.367 ± 0.263 (0.002–0.825)	0.428 ± 0.221 (0.032–0.869)

All traits were measured in mm

biguttulus was 56.9° ± 10 (36.9–75), in *rufus* it was 57.3° ± 10 (35.5–74.8), and in *parallelus* the angle was 60.2° ± 9.9 (39–76.4) with no significant differences among them (Fig. S13). Hence, response vectors were deflected to a similar magnitude in all species. We also calculated angles between predicted response vectors of different species to evaluate if they were deflected in a similar direction. The mean angles between the response vectors of *biguttulus* and *rufus* was 51.5° ± 19.7 (19.2–93.3), between *biguttulus* and *parallelus* was 53.9° ± 21.2 (19.1–102.6) and that between *rufus* and *parallelus* was 49.6° ± 20.2 (17.6–94.3). The direction of deflection was thus markedly different in all pairwise comparisons (all > 49°). However, the random skewer analysis was associated with large uncertainties. Even randomized samples from the posterior distributions of response vectors produced average angles of 38.3° ± 18.1 (12.2–80.8) for *biguttulus*, 38.1° ± 18.7 (12.4–83.6) for *rufus*, and 37.1° ± 21.6 (10.4–95) for *parallelus*.

Flury hierarchy analysis

The Flury hierarchy analysis for the three matrices reveal that the **G** matrices are not equal or proportional, or share any CPCs, as the best fitting model is the one of unequality or heterogeneity. Models with CPCs yielded significantly worse fits with AIC increasing almost steadily with the number of CPC being added. Hence, the matrices do not show stability in terms of the stability of eigenvectors.

Genetic covariance tensor analysis

Genetic covariance tensor analysis estimates (co)variation among elements of **G** across species. In our study with three species-specific **G** matrices, the maximum possible number of nonzero eigenvalues of the genetic covariance tensor is 2. The first eigentensor $\mathbf{E}_1$ explains 71% of the variation among **G** matrices (Fig. 3, Table S9). The 95% CI of the eigenvalues did not overlap between observed and randomized **G** matrices for both the eigentensors $\mathbf{E}_1$ and $\mathbf{E}_2$, illustrating significant variation among matrices. Further, the eigenanalysis of the eigentensor showed that the first eigenvector $\mathbf{e}_{11}$ of $\mathbf{E}_1$ explains 74% and first eigenvector $\mathbf{e}_{21}$ of $\mathbf{E}_2$ explains 40% of variation in eigentensors. Wing length loads on both eigenvectors in particular in the mean-standardized analysis (Table S9). This suggests that wing length contributes most to the major axis of variation among **G** matrices compared with other traits, and that the matrices must have diverged along wing length. The second eigenvector $\mathbf{e}_{22}$ of the second eigentensor $\mathbf{E}_2$ captures 39% of the variation in $\mathbf{E}_2$. Hence the two eigenvalues of substantial size suggests that the independent genetic change represented by $\mathbf{E}_2$ occurs mainly in two genetically independent trait combinations (Table S9) (Hine et al. 2009). The

Table 2 Between-trait correlations in males and females of *Chorthippus biguttulus*, *Gomphocerippus rufus*, and *Pseudochorthippus parallelus* along with their SE and 95% CI

	Femur	Wing	Antenna	Lobe	Eye
C. biguttulus					
Femur	0.595 ± 0.321 (−0.306 to 0.952)	0.149 ± 0.408 (−0.673 to 0.845)	0.156 ± 0.362 (−0.614 to 0.728)	0.454 ± 0.384 (−0.455 to 0.946)	0.405 ± 0.386 (−0.522 to 0.924)
Wing	0.418 ± 0.298 (−0.333 to 0.857)	0.474 ± 0.282 (−0.182 to 0.903)	−0.277 ± 0.304 (−0.751 to 0.451)	0.406 ± 0.301 (−0.248 to 0.905)	0.248 ± 0.316 (−0.382 to 0.841)
Antenna	**0.468 ± 0.185 (0.065–0.778)**	0.149 ± 0.266 (−0.400 to 0.602)	0.337 ± 0.227 (−0.133 to 0.751)	−0.242 ± 0.348 (−0.772 to 0.554)	−0.165 ± 0.315 (−0.688 to 0.549)
Lobe	**0.726 ± 0.181 (0.258–0.946)**	0.573 ± 0.261 (−0.096 to 0.924)	0.376 ± 0.220 (−0.120 to 0.751)	**0.663 ± 0.231 (0.071–0.952)**	**0.705 ± 0.158 (0.330–0.943)**
Eye	**0.573 ± 0.196 (0.105–0.859)**	0.649 ± 0.200 (0.178–0.917)	0.157 ± 0.216 (−0.290 to 0.553)	**0.735 ± 0.151 (0.379–0.919)**	**0.830 ± 0.099 (0.590–0.960)**
G. rufus					
Femur	**0.681 ± 0.190 (0.191–0.923)**	0.002 ± 0.438 (−0.768 to 0.816)	0.280 ± 0.252 (−0.278 to 0.708)	**0.505 ± 0.218 (0.028–0.854)**	0.291 ± 0.301 (−0.369 to 0.769)
Wing	−0.113 ± 0.471 (−0.829 to 0.761)	0.274 ± 0.409 (−0.660 to 0.863)	0.017 ± 0.347 (−0.592 to 0.691)	−0.155 ± 0.447 (−0.796 to 0.793)	−0.230 ± 0.466 (−0.853 to 0.776)
Antenna	**0.416** ± 0.195 (−0.042 to 0.718)	0.079 ± 0.351 (−0.572 to 0.717)	**0.483 ± 0.200 (0.057–0.808)**	0.130 ± 0.274 (−0.411 to 0.638)	0.103 ± 0.293 (−0.484 to 0.639)
Lobe	**0.721 ± 0.140 (0.375–0.906)**	−0.109 ± 0.470 (−0.845 to 0.795)	0.345 ± 0.192 (−0.086 to 0.679)	**0.631 ± 0.186 (0.179–0.898)**	**0.572 ± 0.200 (0.108–0.867)**
Eye	**0.529 ± 0.176 (0.107–0.792)**	−0.036 ± 0.434 (−0.764 to 0.768)	0.373 ± 0.178 (−0.024 to 0.692)	0.791 ± 0.080 (0.604–0.913)	**0.727 ± 0.149 (0.355–0.918)**
P. parallelus					
Femur	**0.711 ± 0.199 (0.210–0.943)**	0.645 ± 0.279 (−0.152 to 0.931)	**0.656 ± 0.221 (0.040–0.896)**	0.670 ± 0.316 (−0.287 to 0.943)	0.291 ± 0.301 (−0.369 to 0.769)
Wing	**0.594 ± 0.199 (0.161–0.902)**	0.566 ± 0.259 (−0.092 to 0.910)	0.561 ± 0.287 (−0.226 to 0.906)	0.643 ± 0.318 (−0.223 to 0.948)	−0.230 ± 0.466 (−0.853 to 0.776)
Antenna	0.339 ± 0.205 (−0.082 to 0.747)	**0.510 ± 0.189 (0.088–0.823)**	0.393 ± 0.265 (−0.219 to 0.810)	0.589 ± 0.307 (−0.236 to 0.925)	0.103 ± 0.293 (−0.484 to 0.639)
Lobe	0.555 ± 0.332 (−0.397 to 0.928)	0.415 ± 0.330 (−0.419 to 0.881)	0.236 ± 0.313 (−0.456 to 0.758)	0.522 ± 0.385 (−0.534 to 0.937)	**0.572 ± 0.200 (0.108–0.867)**
Eye	0.260 ± 0.462 (−0.755 to 0.896)	0.205 ± 0.428 (−0.686 to 0.839)	0.108 ± 0.369 (−0.647 to 0.729)	0.226 ± 0.443 (−0.693 to 0.867)	**0.727 ± 0.149 (0.355–0.918)**

Male correlations are shown in the lower triangle and females correlations in the upper triangle.The diagonals (highlighted by grey shading) contain the cross-sex correlations. Correlations with CI that do not overlap zero are shown in bold

projection of the eigenvectors onto the observed **G** matrices showed that the change in genetic variance represented by $\mathbf{e}_{11}$ and $\mathbf{e}_{21}$ is mostly attributable to *parallelus*. Though the credibility intervals overlap, there is a trend especially for $\mathbf{e}_{11}$ (Fig. 3). Hence, along $\mathbf{e}_{11}$, there is differentiation among *parallelus* on the one hand and the species pair *biguttulus*/*rufus* on the other, and this change in V_A probably bears the signature of divergence of *parallelus* from the latter two.

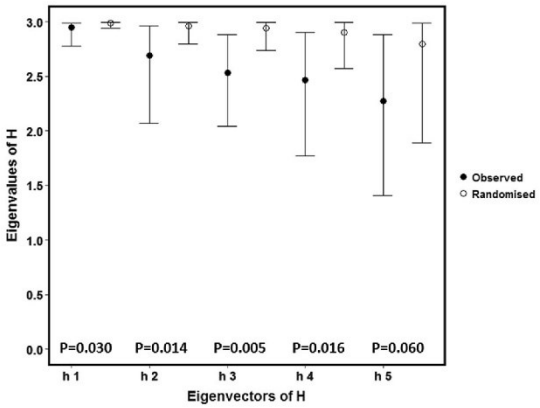

Fig. 2 Krzanowski's subspace **H** for the comparison of **G** matrices among three species of grasshoppers. The *x*-axis denotes the five eigenvectors $\mathbf{h}_1$–$\mathbf{h}_5$ of the **H** matrix and the *y*-axis denotes the eigenvalues of **H**. Filled symbols show empirical estimates with 95% CI and open symbols show randomized values. *P* values denote the proportion of randomized values that show equal or lower values than empirical estimates (incorporating variability in both empirical and randomized values)

Divergence analysis

Species divergence can be summarized by the divergence matrix **D** in mean trait values (Table S6). We used eigenanalysis of **D** to summarize the main axes of divergence. The first two eigenvalues of **D** explained the majority of species divergence (93% and 7%, respectively). Wing length loaded heavily on the divergence axis 1 and antenna and femur on the axis 2 (Table S7). We projected contemporary genetic variation as summarized by **G** into the main axes of historical divergence space (Fig. 4). *Rufus* is most strongly aligned with the second principle component axis that captures mostly the differences *rufus*/*biguttulus*, whereas *biguttulus* has a less pronounced alignment with least structure, which is indicative of the weak genetic correlations. Contemporary genetic variation in *parallelus* is oriented away from divergence axis 1, but the breeding value ellipse illustrates high genetic correlations.

Discussion

We estimated and compared the genetic (co)variance structure of 10×10 **G** matrices consisting of five morphological traits expressed in both sexes in three grasshopper species. The subspace analysis shows significant difference in dominant subspaces among **G** matrices. The random

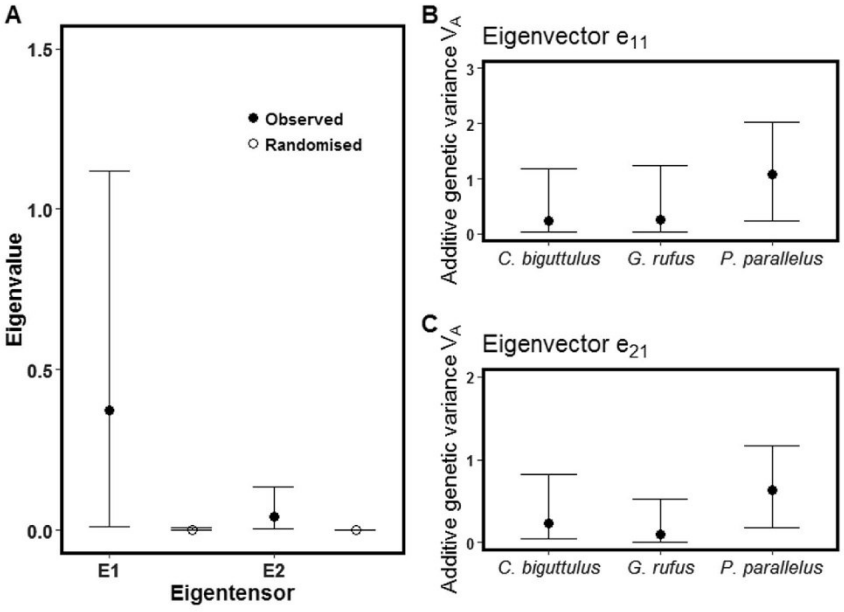

Fig. 3 Genetic covariance tensor analysis. **a** Variance in variation among **G** matrices as explained by the two eigentensors $\mathbf{E}_1$ and $\mathbf{E}_2$ of the covariance tensor $\boldsymbol{\Sigma}$ along with credibility intervals. Observed values (filled symbols) were compared with values after randomization (open symbols). Eigenanalysis of each **E** identifies the major axis of genetic variation among **G** matrices. Figures show the amount of additive genetic variance in each species along **b** the major axes $\mathbf{e}_{11}$ of $\mathbf{E}_1$ and **c** the major axis $\mathbf{e}_{21}$ of $\mathbf{E}_2$

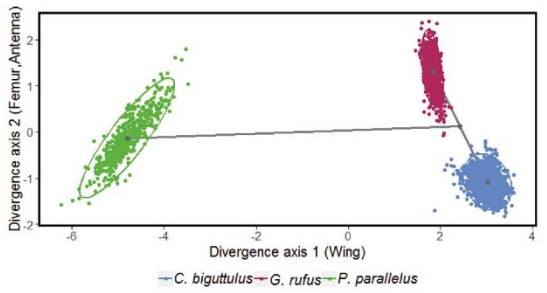

Fig. 4 Contemporary genetic variation projected into divergence space among the three species of grasshoppers. The axes show the first two principal components of the species divergence matrix **D** (Table S7). Breeding values of the three species are plotted onto the same plane. Ellipses around breeding values show 95% confidence level. Lines indicated the main direction of divergence as they connect the center of the more closely related *Chorthippus biguttulus* and *Gomphocerippus rufus* as well as the midpoint of these two species to the center of *Pseudochorthippus parallelus*

skewer analysis also suggests marked differences in deflection angles, although this is accompanied by large uncertainties. In line with this, the Flury analysis identifies no shared principle components. The tensor analysis indicates difference in shape and orientation of **G** matrices with most standing genetic variation in the direction of **G** matrix divergence in *Pseudochorthippus parallelus*. It also identifies wing length as the most influential trait. The same pattern is also seen in the divergence analysis. Furthermore, the divergence analysis illustrates that the main axis of species divergence is only partly aligned with genetic variation within species. It is mostly *Gomphocerippus rufus* that clearly shows most genetic variation in the direction of divergence from *Chorthippus biguttulus*, while genetic variation in *parallelus* is oriented away from the direction of divergences from the other two species. Overall, these matrix comparisons illustrate rather substantial differences in **G** matrices. The comparisons reveal a phylogenetic signal that related species are more similar in their **G** matrices, that alignment with the main axis of divergence is prominent only in the youngest species pair, and finally they identify wing length as a key trait that contributes substantially to matrix differences.

The motivation for the comparative analysis builds on the assumption that the divergence axis is an indicator of past selection (Schluter 1996). Under these conditions, the comparison of species divergence and standing genetic (co) variation allow insights into the alignment of the main axis of **G** with the direction of selection. There are three reasons why the main axis of genetic variation, $\mathbf{g}_{max}$, might be aligned with the main axis of selection. First, divergence might be faster and more efficient in the direction of $\mathbf{g}_{max}$ and that the divergence axis therefore represents a compromise between the forces of selection and the influence of genetic covariance (axis of least genetic resistance hypothesis, (Schluter 1996)). Second, the genetic covariance structure might be shaped by correlational selection to align with the dominant direction of selection (Lande and Arnold 1983; Phillips and Arnold 1989; Sinervo and Svensson 2002). Finally, the axis might be aligned by chance.

Despite these reasons for alignment, our observation does not support this. Instead, the results suggest that any alignment is a matter of temporal scale. There is some indication for alignment between *rufus* and *biguttulus*, in particular in the genetic covariance structure of *rufus*. On a larger temporal scale, the more distantly related **G** matrix of *parallelus* is poorly aligned. This is expected for example if the direction of selection might have fluctuated since the split between species and we do not know if it is the more recent history of selection that dominates the shape of **G** or the average long-term pattern of selection (Steppan et al. 2002; McGuigan 2006; Careau et al. 2015). Previous studies on within-species comparisons that illustrate differences in **G** (see 'Introduction' section) suggest that changes in **G** can be strongly affected by the recent past. On the other hand, there are also studies on congeneric species that suggest alignment over longer timescales (Schluter 1996). Only additional empirical results on different species will allow a better understanding of which trends predominate and how the peculiarities of every individual system affect the outcome.

We found evidence for a phylogenetic signal in the **G** matrix divergence among species, such that the **G** matrix for the most distantly related species is the least similar. Such a phylogenetic signal is not always observed in comparative **G** matrix analyses. The **G** matrices of crickets, for example, do not diverge according to phylogenetic relatedness and are overall rather similar (Bégin and Roff 2004). Specifically, we found alignment with the main axis of divergence only in the youngest species pair, but not with respect to a more distantly related species. Previous studies have reported an alignment of the divergence with the genetic variation in deeply diverged sets of Anolis lizards (McGlothlin et al. 2018) as well as in a much younger radiation of ecotypes in plants (Walter et al. 2018). Overall, there is still little data on whether **G** matrix similarity reflects phylogenetic relatedness, but there is some evidence for alignment of divergence with genetic covariation. Our data suggests that both **G** matrix shape and alignment with the axis of divergence are a matter of temporal scale.

Wing length turned out to be a particularly influential trait in our analyses. It is one of the longest structures that we have included in our study (Table S1) and it might seem intuitive to assume that the size itself is causing the dominance on **G** matrix divergence. However, the same patterns are also present in the mean-standardized analysis, illustrating that its influence is not only due to the scaling of the

variance with the mean (Houle 1992), but that variation in wing length is large even when accounted for its size. The length of the wings is among the most variable characters of grasshoppers, with long wings in some species (such as *biguttulus*, but also others with even longer wings) and wings significantly reduced in others (such as *parallelus*). Wing length is also highly variable within species, and tends to show substantial sexual dimorphism. Males typically have longer wings and they use their forewings for stridulation (Uvarov 1966). Females do not produce advertisement songs and their wing length is often reduced compared with males. Finally, wing length is sometimes polymorphic within populations independent of sex (Harrison 1980; Roff 1986a; Roff and Fairbairn 2007), further illustrating that it varies independent of other morphological traits. Wing length thus seems to respond quickly to selection and is genetically decoupled from other morphological traits.

We used four formal matrix comparison methods and projection into divergence space to compare matrices. Although other methods exist (Roff et al. 2012; Teplitsky et al. 2014), our analysis represents a very comprehensive exploitation of matrix comparison methods. Overall, results were consistent across methods, but there are also relevant differences. The random skewer analysis was indicative of differences among **G** matrices, but was accompanied by large sampling variation suggesting relatively low power. Low power of the random skewer analysis has also been reported in simulation studies (Teplitsky et al. 2014). On the other hand, the Flury analysis seems to indicate rather substantial differences in the covariance structure of the matrices, with none of the matrices having any CPCs. It seems possible that the Flury analysis overemphasizes differences between matrices. The subspace and the tensor analysis seem to be most nuanced, illustrating significant differences without dismissing the similarities that do exist among matrices. Both methods also suggest that *parallelus* contributes most to these differences. The tensor analysis furthermore identifies the traits contributing most to differences among **G** matrices.

Our analysis is one of the first of its kind to show the distribution of breeding values in divergence space (but see (McGlothlin et al. 2018)). Phylogenetically, *biguttulus* is more closely related to *rufus* than *parallelus* (Vedenina and Mugue 2011). Using average trait values of the more closely related species pairs implicitly applies an ancestral state reconstruction that may be very simple, but offers a point of comparison for the more distantly related species. The divergence analysis shows that the two ellipses for *rufus* and *biguttulus* are aligned in the same direction, compared with *parallelus*, which is oriented away from the divergence axis. Phenotypic divergence is used as a surrogate for past selection (Schluter 1996), although it is evidently the consequence of selection and drift and also influenced by the (unknown) ancestral shape of **G**.

Conclusions about **G** matrix similarities are also influenced by the choice of traits. In our analysis, we treated traits in females and males as sex-specific traits. As expected, correlations among traits expressed in the two sexes produce rather strong cross-sex genetic correlations and hence structure in **G** that is similar across species. The decision to treat traits as sex specific thus influences the amount of structure in **G** and the similarity in comparisons. Furthermore, our analysis is focused on bilateral traits. We treated the two sides as replicate observations that were effectively averaged for the same trait as a tool to reduce measurement error. However, we could have treated left and right sides as distinct traits which would have created strong structure in **G**, since genetic integration almost certainly produces strong genetic correlations among sides and again this structure would be shared among species. The choice to either treat sides as distinct or the same trait most likely influences the outcome of **G** matrix comparisons.

Statistical power is always a concern when estimating genetic covariation. We here report **G** matrices based on decent sample sizes of around 900–1100 for two of the species, but clearly less for the third species. However, our results also illustrate that it is not only the sample size that determines the outcome. We describe biologically expected patterns in the structure of the **G** matrix that are unlikely to be produced with insufficient data. In particular, we find a ranking of genetic correlations being highest for the same traits expressed in the two sexes, lower for genetic correlations among traits within sexes and lowest for correlations among traits among sexes. Furthermore, we show that the magnitude and precision of estimates depend on the magnitude of both heritabilities and of genetic covariation (Figs. S8 and S9, Table S11). Hence the structure of genetic variation partly determines the precision of estimates independent of sample size. The structure of genetic variation is not under the experimenter's control, making it difficult to perform power analysis if the magnitude of genetic (co) variation is unknown.

Of special interest in **G** matrix analyses are the cross-sex genetic correlations, since they are relevant to the evolution of sexual dimorphism. Low cross-sex correlation in homologous traits suggests that sex-specific selection is at work to a certain degree (Chenoweth and Blows 2004; Day and Bonduriansky 2004; Bonduriansky and Rowe 2005). This helps the sexes to achieve their sex-specific optima (Bonduriansky and Chenoweth 2009). A review of cross-sex correlations reported a mean r_{MF} of 0.80 for morphological traits predicted when there is no sexual dimorphism (Poissant et al. 2010). Our study shows that the evolution of sexual dimorphism might be somewhat impeded by covariation, although the constraint is not absolute as is

illustrated by the non-perfect correlations (average 0.54) and the existence of sexual dimorphism in all traits. Wing length and antenna length are the two traits with lowest r_{MF} (average 0.44 and 0.40, respectively). Wings are involved in sound production in grasshoppers and show marked sexual dimorphism in many species (Roff 1986a; Gäde 2002; Rosetti and Remis 2018) including some crickets and bush crickets (Roff 1986b; Roff and Fairbairn 1993; Heidinger et al. 2018). The antennae are also involved in courtship display in particular in *rufus* (Riede 1986). Both traits are thus likely to be the target of sex-specific selection and even tend to show reverse sexual dimorphism by being larger in otherwise smaller males (Table S1).

Besides genetic variation, we find substantial amounts of individual identity effects illustrating a large amount of phenotypic flexibility. This is not too surprising for grasshoppers that often show strong developmental plasticity, for example in temperature-dependent variation in overall body size and life-history traits (Hinks and Erlandson 1994; Willott and Hassall 1998). Despite the common garden situation, there is always some heterogeneity in conditions such as local temperature, food quality, and competition that might affect the development. Idiosyncratic effects might arise in particular if particular environmental stages are sensitive to have a disproportional effect on final body size. The first nymphal stages in particular seem to be quite variable in molting times, activities, and growth, possibly representing a critical stage during development.

An auxiliary finding of our study is the spontaneous occurrence of macropterous *parallelus* in a population of exclusively short-winged adults. Phenotypic plasticity in wing length is well documented in grasshoppers (Roff and Fairbairn 2007; Forsman 2015). The fact that macropterous individuals were neither clustered in genetic families nor in rearing cages suggests that neither a simple genetic mechanism nor any strong environmental trigger produces macropterous individuals. Crowding has been implicated in macropterism in a number of Orthopterans (Harrison 1980). Although not designed for this purpose, our data does not suggest a role of crowding in wing dimorphism of *parallelus*.

Overall, the structure of genetic variation is remarkably variable among the three species. Notably, however, the **G** matrices of *biguttulus* and *rufus*, the more recently diverged species pair, are better aligned to the direction of divergence in morphology, while the more distantly related *parallelus* is not. Wing length is contributing most to these differences and wing length is also the trait that is most variable between and within species, including variability among the sexes. It is thus the trait that is most affected by both population-specific and sex-specific selection. This might indicate a role of selection shaping **G** matrix differences. Our analysis thus illustrates that the structure of the **G** matrix can be variable when assessed over longer evolutionary timescales even for largely conserved morphological traits.

Data archiving

Data available from the Dryad Digital Repository: https://doi.org/10.5061/dryad.rjdfn2z5x.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Manuscript II: G matrix of songs

Title: Multivariate genetic architecture of songs in Gomphocerine grasshoppers

Multivariate genetic architecture of songs in Gomphocerine grasshoppers

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Abstract

Evolutionary trajectories of quantitative traits are largely shaped by the underlying genetic architecture of such traits. Traits are often genetically correlated with each other and the resulting covariation strongly influences the traits' response to selection. The additive genetic variance-covariance matrix **G** efficiently summarizes the amount of heritable (co)variation. In case of secondary sexual characters, sexual selection acts on traits which are usually correlated and thus prompting to undertake a multivariate approach by estimating **G**. We analyze five song traits of male grasshoppers to estimate 5x5 **G** matrices in two species. We used a comparative quantitative genetics approach to assess similarities and dissimilarities between those **G** matrices. We found mostly high heritabilities in *Chorthippus biguttulus* with strophe duration (0.54) and syllable duration (0.50) being highly heritable, and maximum amplitude, a trait under sexual selection, showing low heritability (0.26). The overall heritabilities in *Gomphocerippus rufus* were low, with highest values for strophe duration (0.30). We also find tight negative genetic (-0.65) as well as phenotypic correlation (-0.48) between strophe duration and maximum amplitude showing length of song and loudness achieved are strongly dependent upon each other. **G** matrices thus differed significantly between the two species in both orientation and shape. The stronger genetic correlations in *C. biguttulus* form a more constrained genetic architecture compared to *G. rufus*. Our results reveal that the **G** matrices of male ornamental song traits have diverged over time with their trajectories mostly influenced by selection.

Keywords: additive genetic correlations, comparative quantitative genetics, courtship song, sexually selected traits, Orthoptera, sexual dimorphism, genetic constraint, multivariate trait evolution

Introduction

The genetic architecture of quantitative traits influences their evolutionary trajectories in phenotypic space (Lynch & Walsh, 1998). One way to study the genetic architecture is to scrutinize the structure of additive genetic variances and covariances of traits (Hansen, 2006). Genetic covariances stem from genetic coupling of traits due to linkage disequilibrium or pleiotropy. Since such coupling is widespread, traits seldom evolve in isolation (Lynch & Walsh, 1998, Blows, 2007). Hence a multivariate approach to studying evolutionary change becomes more pertinent than univariate analyses (Lande, 1980a, Blows, 2007, Walsh & Blows, 2009). The matrix of additive genetic variance and covariance **G,** which provides a statistical summary of the amount of heritable genetic variation and covariation among traits, elegantly captures the multidimensional genetic architecture of correlated traits (Lande, 1979, Lande, 1980a). The **G** matrix is essential for predicting the multivariate response to selection of complex traits as is evident from the multivariate Lande equation, which states that the change in mean trait values (response to selection), **Δz**, is a function of the **G** matrix and the vector of linear selection gradients, **β**; $\Delta \mathbf{z} = \mathbf{G}\boldsymbol{\beta}$ (Lande, 1979, Lande & Arnold, 1983). The fact that selection acting on a trait will also produce an evolutionary response in genetically correlated traits is apparent from this equation (Lynch & Walsh, 1998). As adaptive evolution is essentially a multivariate process, a univariate approach not only limits the interpretation, but can sometimes be misleading (Walsh & Blows, 2009).

The importance and implications of the **G** matrix in multivariate evolution becomes even more intriguing when we consider the evolutionary trajectories of secondary sexual traits (Lande, 1980b). Sexual selection stems from the variance in reproductive success due to intrasexual competition or intersexual mating preference, though these mechanisms can be difficult to tell apart (Lande, 1980b, Arnold, 1983). The evolution of extravagant ornamental traits in one sex (often males), and a preference for such traits in the other sex (often in females), lie at the heart of sexual selection. Both the ornamental and preference traits often show continuous variation and are polygenic in nature (Lande, 1980b, Mead & Arnold, 2004). Furthermore, female choice often acts on a suite of male traits rather than individual traits in isolation (Prokop & Drobniak, 2016). Because of such kind of inheritance pattern, game theoretic and two locus population genetic models miss out on the intricate evolutionary dynamics of sexually selected traits (Reid, 2014). Quantitative genetics provides an elegant framework for studying the importance of direct and indirect selection on preference traits and also for the implications of existing theories (for example, good genes, condition dependence, etc.) in the evolution of sexually selected extravagant traits (Rowe & Houle, 1996, Mead & Arnold, 2004, Reid, 2014).

One of the perplexing questions in evolutionary biology is the paradox of 'lek'. When there is continuous sexual (or natural) selection acting on preferred traits, the prediction is that the genetic variance of such traits gets eroded (Pomiankowski & Møller, 1995). The fact that genetic variation is still maintained in sexually-selected traits and in mating preferences for such traits, is known as the lek paradox (Kirkpatrick & Ryan, 1991). Empirical studies find that there is, on average, as much genetic variance for sexually selected traits as for non-sexually selected trait (Prokuda & Roff, 2014). When we classify non-sexually selected traits into morphological, behavioral and physiological traits, heritabilities for behavioral and physiological traits are generally low (possibly partly due to relatively large measurement error). There are several theories like genic capture and condition dependence, mutational variance, or epigenetic resolution as possible explanation for this paradox (Pomiankowski & Møller, 1995, Rowe & Houle, 1996, Bonilla et al., 2016, Dugand et al., 2019), but none of these provide a full proof explanation for the problem (Blows et al., 2004). In fact, simulations show that condition-dependence seems effective for single traits, but insufficient for multivariate cases (Hine et al., 2004, Van Homrigh et al., 2007).

A common assumption of models of sexual selection is the constancy of **G** matrix over time (Mead & Arnold, 2004). The argument for such an assumption is that erosion of genetic variation by selection is compensated by inputs from mutation and recombination (Lande, 1980a). But whether such an argument holds true for sexually selected traits is debatable. As ornamental traits are continuously under selection, the **G** matrix is bound to evolve. However, the **G** matrix can be stabilized in large populations by correlational selection and recurrent pleiotropic mutations (Jones et al., 2003). In small populations, the **G** matrix will evolve by both selection and genetic drift, the latter causing primarily proportional changes in **G** (Roff, 2000). Empirical studies are needed to provide fruitful insights into the stability of **G** over time. Especially, comparative quantitative genetics can help to deduce when and under what conditions **G** changes (Steppan et al., 2002). For sexually selected traits, empirical studies can show the patterns of changes in the structure of **G** depending on the type of trait (Prokuda & Roff, 2014).

The enormous number of male display traits reported in studies and the high heritability of single preferred traits, indicate that such traits might respond fast to selection (Pomiankowski & Møller, 1995, Rowe & Houle, 1996). In fact, high heritability of traits was reported both within population (Pomiankowski & Møller, 1995, Brooks & Endler, 2001) and among populations (Hughes & Leips, 2006). But studies reporting how female choice itself changes the evolutionary trajectories of sexually selected traits are rare, and those studies that are available, at least partly show no response to sexual selection (Merilä et al., 2001, Kruuk et al., 2002, Hall et al., 2004). High heritability thus does not simply translate to high evolvability (Holman & Jacomb, 2017). One reason for such an

evolutionary limit to sexually selected traits might be the distribution of genetic variance in phenotypic space (McGuigan et al., 2008). There can be little or no genetic variance in multivariate trait combinations of **G** along which selection acts resulting in little or no response to selection. Hence, whether **G** is stable for such traits is essentially an open question that has to be addressed empirically.

An interesting outcome of sexual selection is that it can drive speciation (Lande, 1981, Panhuis et al., 2001, Kirkpatrick & Ravigné, 2002, Van Doorn et al., 2009). Sexual selection can cause rapid divergence between populations by evolutionary change in secondary sexual traits and preference causing prezygotic isolation, hence speeding up speciation (Panhuis et al., 2001). This poses an important evolutionary question on the divergence of **G** matrix for courtship display traits in closely related species. **G** can evolve for ornamental traits due to strong sexual selection, but the structure of **G** can also influence how species diverge. Some studies have compared **G** for male sexually selected traits among populations (Ashman, 2003, Pascoal et al., 2017), or within populations (Blows et al., 2004, Hine et al., 2004, Van Homrigh et al., 2007, McGuigan et al., 2008, Welch et al., 2014, Holman & Jacomb, 2017). But there is a dearth of studies comparing multivariate genetic architecture of male display traits among species (but seeRoff et al., 1999). Such comparative studies could provide insights to the stability or evolution of **G**, especially for sexually selected traits.

Male calling song in insects is an example of such a suite of polygenic traits which is under multivariate sexual selection (Higgins & Waugaman, 2004, Bentsen et al., 2006, Oh & Shaw, 2013, Ower et al., 2013). Besides being central to female preference, calling songs also help in mate recognition (Roff et al., 1999). Males (and sometimes females) produce elaborate songs as courtship displays and females choose males based on specific song characteristics. It is particularly fascinating in Orthopterans where morphologically similar sympatric species produce markedly different, species-specific songs (Charalambous et al., 1994, Tregenza et al., 2000). The genetic covariance structure of song traits such as duration, pulse rate, carrier frequency etc. can provide deeper understanding about the evolutionary trajectory of mating songs. There are several behavioral studies that report female preference for particular song traits in Orthopterans (Butlin et al., 1985, Charalambous et al., 1994, Klappert & Reinhold, 2003, Champagnon & Cueva del Castillo, 2008). However, unless there is additive genetic variance for such traits, there will be no evolutionary response even in the presence of strong female choice (Champagnon & Cueva del Castillo, 2008). Univariate heritability estimates for song traits have been reported for Orthopterans (Butlin & Hewitt, 1986a, Hedrick, 1988, Webb & Roff, 1992, Simmons, 2004, Champagnon & Cueva del Castillo, 2008), but exploration of multivariate genetic architecture is completely lacking.

Here, we estimate and compare **G** matrices of song traits of two species of grasshoppers from the subfamily Gomphocerinae, a clade of grasshopper with worldwide distribution of 230 species (Cigliano et al., 2017). Grasshoppers produce songs by rubbing a stridulatory file of the femur on a raised vein of their fore wings (Uvarov, 1966). We studied two rather closely related species within this clade, *Chorthippus biguttulus* and *Gomphocerippus rufus*, which show 4.8% mitochondrial sequence divergence (Contreras & Chapco, 2006). The two species are morphologically similar, but there are striking differences in the complexity of song structure. G. *rufus* has a long and complex courtship song with a single strophe, accompanied with visual display by swaying of club-shaped antennae, whereas *biguttulus* has songs with multiple strophes (3-5) separated by short pauses. As songs are vital in mate recognition, we expect a divergence in **G** matrices along these song traits. Rapid speciation in courting species such as *G. rufus* and *C. biguttulus* has been suggested to be a product of sexual selection (Vedenina & Mugue, 2011). Hence estimating and comparing the genetic covariance structure of song traits in species with such an intricate phylogenetic history may give us a better understanding of their pattern of divergence. Such a comparative study is stimulating as it enlightens us whether these diversified song structures differ in their genetic architecture.

We analyzed five song traits, duration of songs, pulse rate, maximum amplitude, carrier frequency and onset accentuation, to estimate and compare their genetic covariance structure between the two grasshopper species. Firstly, we estimated heritabilities of these traits, and also the proportion of individual and song session variance contributing to the total phenotypic variance of the traits. Secondly, we analyzed the between trait covariations as an indication of multivariate genetic constraint. Finally, we compared the **G** matrices with various matrix comparison methods to understand the degree of similarity among matrices.

Materials and Methods

Study organisms

We analyzed male calling songs of two Acridid grasshoppers, *Chorthippus biguttulus* and *Gomphocerippus rufus* (in the following we refer to the two species by species name only). Males of these two species have distinct song structures. When attracted to songs of particular males, females of *rufus* and *biguttulus* sing back in response (response stridulation) (Butlin & Hewitt, 1986b), but female songs are much softer and shorter than male songs. The two species, like most grasshoppers, are sexually dimorphic in morphology (Chakrabarty & Schielzeth, 2020). We collected *biguttulus* from Bielefeld, Germany (52° 1’ N; 8° 28’ E), and *rufus* from Tubingen, Germany (48° 30’ N; 9° 4’ E). *Biguttulus* inhabits dry to mesic grasslands, whereas *rufus* occurs on semi-open slopes with tall

grasses. We collected final instar nymphae from the field and kept them in large housing cages until final molt. Each adult was marked with a numbered bee tag. Virgin adults were kept separately in large housing cages (47.5 x 47.5 x 90 cm^3) until mating.

Breeding design

We used a paternal half-sib breeding design to study the genetic covariance structure of songs in grasshoppers. Virgin females were kept in individual mesh cages (22 x 16 x 16 cm^3) and one male was mated to two females forming a half-sib family. Males were switched between cages every 2-3 days until they died. Upon death of females, new virgin females were added if the male was still alive. Cages were supplied with sand pots for egg deposition. These pots were sieved once per week to collect egg pods, solid structures that typically contain about 6-10 eggs (Chakrabarty et al., 2019). Each egg pod was then kept in small petri dishes lined with filter paper and was sprayed with water at regular intervals to keep them moist. Petri dishes were kept in refrigerators at a temperature of 0-10°C for at least three months, from October till they were taken out between January and June.

F1 animals

Egg pods were taken out of the refrigerator in five cohorts between January and June and hatching started on 24th January and continued till 11th June. Hatchlings from the same egg pod were kept in the same mesh cage (22 x 16 x 16 cm^3). Cages were provided with vials filled with water and fresh grass. There were 1-10 hatchlings per egg pod with mean ± SD of surviving hatchlings of 3.21 ± 2.28 in *biguttulus* and 4.13 ± 2.35 in *rufus.* Nymphae were kept at a temperature of 25-30°C and at a relative humidity of 40-60%. Upon emergence as adults, subjects were transferred to communal cages (43.5 x 43.5 x 93 cm^3) with a maximum number of 25 individuals per cage. The total number of surviving F1 males were 478 *biguttulus* and 250 *rufus* that had hatched from 223 egg pods in *biguttulus* and 133 in *rufus*. The total numbers of full-sib families were 90 in *biguttulus* and 62 in *rufus* and the total numbers of half-sib families were 65 in *biguttulus* and 44 in *rufus*.

Song recording

Male songs were recorded in custom-build sound recording boxes. Boxes were 28 × 27 × 28.5 cm^3 made from KömaCel panels (KömaCel Panels, Kömmerling, Pirmasens, Germany) and were padded with sound-attenuating sponge (1.8 cm thick) on the sides and top for insulation and the front side of the box was covered by transparent plexi-glass door. Each box contained a microphone (Behringer C-2 condenser microphone, Behringer, Makati City, Philippines) fitted on the top of the cage and a small LED bulb (Ce Led Lamp 6W 3000k 40D Dimmable, Philips, Eindhoven, The Netherlands) at one

top corner. An audio interface (PreSonus AudioBox 1818VSL, PreSonus, LA, USA) was connected to the microphones and transferred the digital signal to a personal computer. Songs were tracked using the software Sound Analysis Pro 2011 (Tchernichovski et al., 2000) in threshold trigger mode at a sampling rate of 44100 Hz with high-pass filtering. Trigger mode means that incoming sound was continuously stored in buffer and the buffer was search for samples exceeding the trigger threshold (7 dB, with at least 100 samples within 2 seconds exceeding the threshold). Recordings were saved as .wav files with 1.5 seconds pre-threshold interval and 2.0 seconds post-threshold interval (max. 3.5 min recording duration). Heating pads were supplied inside boxes to keep the temperature high and a heater was placed inside the room to keep the room warm.

We started recording males 5 days after their final molt and could record up to 16 males in parallel in 16 individual sound recording boxes. Freshly cut grass was placed inside recording boxes as food for males during trials. Each male was recorded twice alone (on different days) and subsequently recorded twice (on different days) with a female present in the sound box. The females were put inside large size steel tea infusers along with fresh grass. Each recording session lasted for one hour. However, here we analyze only songs without presence of females, because recording sessions with females often produced interferences between female and male songs.

Song analysis

We used custom R scripts for analyzing sound files. Soudecibedend file in .wav format were processed using the R packages tuneR (Ligges, 2018), seewave (Sueur et al., 2008) and zoo for calculating rolling mean values (Grothendieck, 2005). Files were loaded with a sampling rate of 44100 and a high-pass filter of 1001 and were converted to amplitude envelopes with window length of 44 sampling points and a window overlap of 50%.

We first automatically identified strophes. An amplitude threshold of 0.02 was used for distinguishing sound from background noise and identified the beginning and end of a strophe as the time windows of 301 samples (approx. 6.8 ms) for which at least two samples exceeded the amplitude threshold. Continuous rolling windows that constantly exceeded these conditions were classified as strophes. Sequences shorter than 0.9 seconds for *biguttulus* and 1.5 seconds for *rufus* were discarded, since they usually represent incomplete songs or occasionally other sound sources. Amplitude envelopes were plotted as oscillograms with their rolling mean values (Figure 1) and all identified strophe cutoffs and were visually inspected for any misidentifications. About 10% of all strophe endings and beginnings were manually curated. For each strophe we quantified strophe duration in seconds.

After strophe identification and manual curation, we used automated scripts to identify syllables within strophes. We first calculated rolling mean amplitude with a window size of 601 samples for *biguttulus* and 301 samples for *rufus* (Figure 1). This allowed use to track changing amplitude levels within strophes. We then calculated the local deviation in amplitude from the rolling mean and identified local minima (tolerance of 10^{-7}). Sections outside the range of 0.02-0.15 seconds for *biguttulus* and 0.05-0.20 seconds for *rufus* were discarded. Identified syllables were numbered in sequences within strophes starting from the last and counting backward in time. We analyzed 20 syllables from near the end of each strophe (syllables 3-22), since syllables at the beginning of a strophe are often soft and variable and final syllables are often different and difficult to identify.

For each syllable we quantified syllable duration (in seconds), minimum amplitude (in dB), maximum amplitude (in dB), amplitude range (in dB) and dominant frequency (in Hz). Syllable duration was converted to syllable rate per second as the inverse of syllable duration. We then combined these measures by strophe and calculated the average and standard deviation (as a measure of variability) of each of these measures across 20 syllables as per strophe.

Chorthippus biguttulus typically sings songs consisting of multiple strophes that increase in amplitude and decrease in duration. We therefore numbered strophes and used gap duration of 7 seconds to separate songs. Within songs, we analyzed the second strophe only. Songs of *Gomphocerippus rufus* are not structured into multiple multiple strophes and we therefore analyzed all strophes for this species. Recordings of individuals with deformed wings or missing hind legs were discarded. In total we extracted data for 9737 strophes (8809 for *biguttulus*, 928 for *rufus*) from 530 males (385 *biguttulus* and 145 *rufus*).

Statistical Analysis

Multivarite linear mixed models were fitted in R 3.6.2 (R Core Team, 2019) under a Bayesian framework using MCMCglmm package (Hadfield, 2010). We fitted multivariate animal models and extracted posterior distribution of variances and covariances. For each trait the basic model was:

$$\mathbf{y} = \boldsymbol{\alpha} + \boldsymbol{Z_1}\mathbf{a} + \boldsymbol{Z_2}\mathbf{i} + \boldsymbol{Z_3}\mathbf{s} + \boldsymbol{Z_4}\mathbf{d} + \boldsymbol{Z_5}\mathbf{b} + \mathbf{e} \qquad (1)$$

where **y** is a matrix of trait values, **a** is the matrix of additive genetic effects, **i** is the matrix of individual identity effects, **s** is the matrix of individual session effects (part of phenotypic variance arising from individuals sharing the same recording session), **d** is the matrix of day effects (individuals were recorded on different days), **b** is a matrix of recording box effects (animals were recorded in different boxes in both the recording sessions), **e** is the matrix of residual errors. All these matrices are of dimensions *N* x *n*, where *N* is the total number of observations and *n* the number of song traits

analyzed). $\mathbf{Z_1}$, $\mathbf{Z_2}$, $\mathbf{Z_3}$, $\mathbf{Z_4}$ and $\mathbf{Z_5}$ are the respective incidence matrices for the five matrices of random effects (additive genetic, individual identity, individual session, day and box respectively). No fixed effects were fitted.

We fitted a five-trait model for both species in order to estimate 5 x 5 **G** matrices (we will refer to the dimension of **G** as *n* in the following). These five traits were strophe duration, average syllable duration, average dominant frequency, average onset accentuation and standard deviation in maximum amplitude. Dominant frequencies and maximum amplitudes were log transformed prior to the analysis. All traits were standardized to unit variance to facilitate model convergence and variances estimated form the model were multiplied by the phenotypic variance in order to recover original scale variances. We estimated all variance components, heritabilities and the covariances between traits from the model.

We used parameter expanded (half Cauchy) priors for fitting the model as these priors are less informative than the regularly used Inverse-Wishart priors (Gelman, 2006). The degree of belief parameter v was set to v = 5 for all the random effects except for the residual, for which it was set to 0.002. The posterior distribution of each model was estimated from 1,100,000 MCMC iterations with a thinning interval of 1,000 and a burn-in period of 100,000. We ran two independent chains per model yielding a total of 2x 1,000 samples form the posterior distribution for each parameter. Model convergence was visually inspected using the trace plots and using Gelman and Rubin diagnostics (Gelman & Rubin, 1992).

Matrix Comparisons

We used three matrix comparison methods that explore different facets of (co)variance matrices. The Krzanowski's subspace analysis determines how similar the subspaces of matrices capturing maximum genetic variance are. The random skewer analysis allows evaluating differences in orientation of **G** matrices. The Flury hierarchy analysis implements a formal assessment of the number of shared eigenvectors.

Krzanowski's common subspace analysis

A way to compare **G** matrices is by comparing subspaces containing maximum genetic variation followed by quantification of overall similarity between submatrices (Blows et al., 2004). Krzanowski's subspace analysis is an approach for comparison of subspaces of matrices and test whether there is an overall similarity of eigenvectors which explain most of the genetic variance across species/populations (Krzanowski, 1979b, Aguirre et al., 2014, Gosden & Chenoweth, 2014). The comparison also shows the amount to which genetic variance shares a similar orientation

between species/populations. The common subspace **H** between the *p* = 2 species is given by (Krzanowski, 1979b):

$$\mathbf{H} = \sum_{t=1}^{p} \mathrm{A}_t \mathrm{A}_t^{\mathrm{T}} \quad (2)$$

Where *t* = 1, …., *p* (*p* = 2, the number of species to be compared), and $\mathbf{A}_t$ contains the subset of k_t eigenvectors of $\mathbf{G}_t$ as columns where *k* is smaller than *n*, the dimension of **G**. The eigenvalues associated with these *k* eigenvectors can assume a maximum value of *p* (Aguirre et al., 2014). We included the first three eigenvectors of **G (**out of five) into the **H** matrix. Eigenvalues of **H** that are equal to *p* indicate similar subspaces among **G** matrices. But if any eigenvalue is less than *p*, then it implies that directions of genetic variation described by that eigenvector differ among matrices, and also that particular eigenvector of **H** cannot be recreated from a linear combination of eigenvectors of **G** as defined by the subspace (Krzanowski, 1979b, Aguirre et al., 2014).

Eigenvectors indicating difference in directions of genetic variation can be used to measure how far they are from each subspace. This can be quantified by the angle $\boldsymbol{\delta_t}$ between each eigenvector of **H** and the subspaces of species *t* (Krzanowski, 1979a, Gosden & Chenoweth, 2014):

$$\boldsymbol{\delta_t} = \mathbf{cos}^{-1}\left\{\sqrt{\mathbf{h_i^T A_t A_t^T h_i}}\right\} \quad (3)$$

When subspace analysis is done under a Bayesian framework using the posterior distribution of **G** matrices, a measure of uncertainty of estimates is obtained (Aguirre et al., 2014). To statistically test whether the observed difference in **G** matrices between the two species is due to sampling variance, we compared the observed data from the subspace analysis to a null model. Randomized **G** matrices were generated from the posterior predictive breeding values of the observed **G** matrices. Any differences in randomized **G** matrices are only due to random sampling. The overlap of confidence intervals between the observed and randomized data was noted. p values were estimated as the proportion of randomized samples that show equal or smaller values than the original MCMC samples. The R code for comparing subspaces was adapted from (Aguirre et al., 2014).

Random skewers analysis

Random skewer analysis stems directly from the tenets of the multivariate Lande equation, $\Delta\mathbf{z} = \mathbf{G\beta}$, where **Δz** is the vector of trait changes and **β** is a vector of selection gradients (Cheverud, 1996, Cheverud & Marroig, 2007, Calsbeek & Goodnight, 2009, Roff et al., 2012). This method primarily compares difference in orientation among **G** matrices. Random linear selection gradients **β** are projected through **G** matrices to generate response vectors **Δz.** The angle between **Δz** and **β** gives the

angle of deflection, whereas the angle between two response vectors from different matrices indicate the difference in direction of deflection when same random selection vectors are applied. Full alignment of average response suggests matrix equality, while misalignment suggests evolutionary significant differences among matrices (Cheverud & Marroig, 2007).

We generated 2000 random skewers from a multivariate normal distribution with uncorrelated axes. These 2000 random skewers were projected through 1000 MCMC samples of each **G** matrix (two chains per species) producing 2000 response vectors. The angle between response vectors can be calculated by

$$\theta = \cos^{-1} \frac{\mathrm{v}_1^{\mathrm{T}} \mathrm{v}_2}{\sqrt{\mathrm{v}_1^{\mathrm{T}} \mathrm{v}_1 . \mathrm{v}_2^{\mathrm{T}} \mathrm{v}_2}}$$

where, $\mathbf{v}_1$ and $\mathbf{v}_2$ are the two response vectors that are compared. As we calculated the angles from each MCMC sample, we extracted a posterior distribution of 2000 angles.

Flury hierarchy analysis

Flury analysis is a method of comparing covariance matrices in a hierarchical way where the hierarchy is built based on the number of principal components shared by the matrices (Flury, 1988, Phillips & Arnold, 1999, Steppan et al., 2002, Cheverud & Marroig, 2007, Roff et al., 2012). Matrices differ from each other in far more nuanced ways than the mere dichotomous state of equality and inequality. Covariance matrices can have all similar eigenvectors but eigenvalues different by a proportional constant, and then the matrices are called proportional. Again the eigenvalues can all be different but the eigenvectors can still be similar, which is called the common principal component (CPC) model. **G** matrices can also share different number of eigenvectors from 1 to *n*-2 where *n* is the dimension of the **G** matrix forming the partial principal component model (PCPC).

Here we used the model–building approach to compare the pair of **G** matrices of two species of grasshoppers (Flury, 1988, Phillips & Arnold, 1999). In this approach a series of models are built starting from inequality to equality including the CPC models. Models were ranked based on their AIC values with lower values indicating a better fit. AIC balances the log-likelihood of a particular model against the number of parameters used to fit that model. We used the R package cpc (Pepler, 2019) to perform the hierarchical analysis using a model building approach. We repeated the test for all the 2000 MCMC samples of the two **G** matrices and obtained a posterior distribution of AIC values. We ranked all the models based on the average AIC.

Results

Heritabilities

We estimated heritabilities and other variance components from the five-trait multivariate animal model (Figure 2). Syllable duration and strophe duration show high heritability in *biguttulus* (0.50 and 0.54, respectively, Table 1). These two traits also show higher heritabilities in *rufus* compared to other traits (0.30 and 0.10, respectively, Table 1). Onset and dominant frequency show low heritabilities in both species.

Genetic correlations

Genetic correlations were overall lower in *rufus* than in *biguttulus.* The strongest correlation in *rufus* was between syllable duration and maximum amplitude (r = 0.44) followed by syllable duration and onset accentuation (r = 0.36) (Table 3). Hence, individuals with breeding values for longer syllables also sang louder and with a higher onset accentuation. In contrast, *biguttulus* showed strong negative correlations between strophe duration and maximum amplitude (r = - 0.65) and onset accentuation and maximum amplitude (r = - 0.40). This shows that individuals with breeding values for longer strophes sang less loud with lower maximum energy levels. The negative genetic correlation between onset and maximum amplitude shows that syllables with high starting amplitude reached low maximum amplitude (were thus less loud). On an average, *biguttulus* shows a tighter correlation than *rufus* with overall more constrained genetic architecture.

Subspace analysis

We worked with the first three eigenvectors of the **G**s to find the dominant subspace, and together they explain 90% variance in *biguttulus* and 85% in *rufus.* We compared the first three eigenvalues of of **H**, and the first two show significantly lower value than that of 2 (the maximum possible eigenvalue with p = 2, Figure 3). The third value showed a clear drop. A value equal to 2 would indicate equality of matrices, but Figure 2 clearly shows differences in orientation of matrices. The angles that quantify closeness between each of the eigenvectors of **H** and the subspaces of the **G** matrices are small (Table 2), though the credibility intervals of the angles overlap. Hence, even when the differences are small, the matrices have diverged in their orientation.

Random skewers analysis

The random skewers analysis shows small differences between **G** matrices of *rufus* and *biguttulus*. We projected 2000 random selection vectors through estimated **G** matrices, and the angle of

deflection was 39.7 ± 11.4 (16.1 - 59.6) for *biguttulus* and 46.5 ± 13.4 (19.9 - 71.3) for *rufus*, indicating no significant difference in average deflection (Figure 4). The angle of deflection between the two response vectors of the two species was quite large 45.8 ± 20.1 (14.8-89.5) and thus illustrates that the direction of deflection was quite different.

Flury hierarchy analysis

The Flury hierarchy demonstrates the inequality of the **G** matrices. There was support neither for equality nor proportionality. The lowest AIC value is for the model of inequality or heterogeneity indicating the best fit. Other CPC models show worse fits with progressively increasing value of AIC as eigenvectors are added under the model building approach of Flury hierarchy (Table2).

Discussion

We estimated and compared the genetic covariance structure of five song traits in two species of Gomphocerine grasshoppers. Of these five traits, strophe duration and syllable duration were highly heritable in *biguttulus*, whereas the heritabilities in *rufus* were overall low. Loudness and length of the strophe showed strong negative correlation in *biguttulus* suggesting loud songs are of comparatively shorter duration. The Genetic correlation structure suggests strong genetic constraint in *biguttulus* with tight correlated responses between pairs of traits. The subspace analysis indicates differences in orientation of the two **G** matrices while the Flury hierarchy analysis showed no common principal components shared between the matrices. Hence, the genetic architecture of song traits in the two species has diverged both in orientation and eigenstructure.

The dissimilarity of genetic architecture of male songs in the two grasshoppers has deeper implications. The matrix comparison methods almost unequivocally point towards very different **G** matrices. Sexual selection has often been invoked as an important tool in speciation (reviewed in (Panhuis et al., 2001)). Songs in grasshoppers are not only under female choice but also play prime role in mate recognition. A minor shift of trait value due to female preference might affect the entire mate recognition system and lead to speciation. It has also been argued that rapid speciation in Gomphocerine grasshoppers is a result of sexual selection (Vedenina & Mugue, 2011). In our study, we also find overall very different **G** matrices in the two species, which means different underlying genetic architecture of traits. However, sexual selection might not act in isolation. We have earlier reported **G** matrix divergence for apparently stable morphological traits in these grasshoppers (Chakrabarty & Schielzeth, 2020). Divergence in genetic architecture of morphology turned out to be dominated by difference in genetic (co)variance involving wing length. Besides locomotion, wings are instrumental in song production and their length is also variable in different species. This might imply

that the non-constancy of **G** matrix between *rufus* and *biguttulus* is an outcome of the interplay between natural and sexual selection.

Songs in Orthopterans are composite traits and some of the components are subject to female choice (Klappert & Reinhold, 2003, Champagnon & Cueva del Castillo, 2008, Stange & Ronacher, 2012). Maintenance of genetic variation in such traits in the face of sexual selection has been an enigma. On a broader scale, the heritability of sexually selected traits tends to decrease as strength of sexual selection increases, but selection is not strong enough to create a difference in heritabilities between the same type of sexually selected and non-sexually selected traits (Prokuda & Roff, 2014). Behavioral traits, for example, have low heritabilities independent of whether they are sexually selected or not. In our study, *Chorthippus biguttulus* shows high heritability in syllable and strophe duration, and moderate heritability in onset accentuation, whereas syllable duration and onset accentuation also show high additive genetic variance. It has been reported that song loudness (amplitude) is under directional selection in this species (Klappert & Reinhold, 2003) and shows a low heritability of 0.15, though there is a dearth of studies reporting selection on ornamental traits which can help us to understand the coupling between heritability of a sexually selected trait and strength of selection. Again, in *Gomphocerippus rufus*, the heritabilities are markedly lower than *biguttulus* except syllable duration, and so are the additive genetic variances. Low heritabilities in song traits have been reported earlier in another closely related Gomphocerine grasshopper, *Chorthippus brunneus* (Butlin & Hewitt, 1986a).

Estimates of pairwise genetic correlation between traits provide us with an understanding of the degree of dependence between traits in their inheritance pattern, as well as the extent of correlated response to selection. In *biguttulus,* most of the traits show tight pairwise correlations and suggest a constrained genetic architecture, while in *rufus* traits are more independent where correlations are not significantly different from zero. On the other hand, maximum amplitude (MA) and strophe duration (SD) show strong negative genetic correlation in *biguttulus*, as does MA and onset accentuation (OA). The phenotypic correlations between MA and SD, and MA and OA are also negative. Hence the longer the strophe, the lower is the MA, as is known in *biguttulus* that the duration of the strophes decreases as the amplitude increases. The negative genetic correlation between MA and OA on the other hand, is a question of being energy efficient. The syllables starting with a high OA are overall less loud and can reach a limited MA. So if an individual male spends higher energy while starting a syllable, it cannot actually produce a louder syllable with high maximum amplitude. Remarkably in *rufus,* we do not find any such negative genetic correlation between any of these traits, but we do find a negative phenotypic correlation between strophe duration and maximum amplitude. This might indicate that there is a similar relation between these

traits as in *biguttulus*, but we might have not been able to detect it due to lower sample sizes in *rufus.*

Maximum amplitude has been identified earlier to be under directional sexual selection in *biguttulus* (Klappert & Reinhold, 2003), with a female preference for louder males. In our study, we get the lowest heritability among all traits for maximum amplitude in both the species. Louder males are easily tracked by the females in the wild, and loudness might be an honest signal as louder males are larger in size. Hence maximum amplitude is one of the major players in sexual selection in grasshoppers. This is also a classic situation where the theoretical prediction that continuous directional selection erodes additive genetic variance in the long term is supported by empirical evidence.

The genetic variance covariance structure of the song traits in *rufus* and *biguttulus* are markedly different. We found that maximum amplitude which has been earlier detected as a trait under sexual selection has also very low heritability. The **G** matrix for biguttulus is more constrained characterized by stronger genetic correlations compared to more independent structure in *rufus*. Our study illustrates that **G** matrices of male ornamental traits have evolved and differentiated over longer evolutionary time scale and might have been largely shaped by sexual selection.

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Tables

Table 1: Genetic variance-covariance matrices for five song traits in *Gomphocerippus rufus* and *Chorthippus biguttulus.* Additive genetic variances is shown on the diagonal (bold), the additive genetic covariance between traits is in the lower off-diagonals, while the upper off-diagonals shows the genetic correlations between traits. Point estimates are accompanied with the ± SE and the upper and lower 95% CI.

G.rufus	Syllable Duration	Strophe Duration	Dominant Frequency	Onset Accentuation	Maximum Amplitude
Syllable Duration	**0.532 ± 0.215**	0.056 ± 0.421	0.024 ± 0.407	0.361 ± 0.323	0.439 ± 0.327
	(0.139-0.983)	(-0.779-0.764)	(-0.739-0.786)	(-0.412-0.85)	(-0.370-0.891)
Strophe Duration	0.02 ± 0.071	**0.069 ± 0.069**	0.224 ± 0.414	0.091 ± 0.423	-0.244 ± 0.435
	(-0.107-0.184)	**(0.000-0.245**)	(-0.699-0.851)	(-0.732-0.825)	(-0.874-0.713)
Dominant Frequency	0.002 ± 0.012	0.004 ± 0.007	**0.002 ± 0.002**	0.048 ± 0.414	-0.102 ± 0.439
	(-0.022-0.03)	(-0.004-0.022)	**(0.000-0.009)**	(-0.744-0.786)	(-0.835-0.782)
Onset Accentuation	0.073 ± 0.068	0.007 ± 0.028	0.001 ± 0.005	**0.08 ± 0.063**	0.20 ± 0.398
	(-0.039-0.217)	(-0.047-0.07)	(-0.010-0.011)	**(0.000-0.229)**	(-0.673-0.856)
Maximum	0.121 ± 0.10	-0.038 ± 0.061	-0.003 ± 0.008	0.20 ± 0.398	**0.165 ± 0.137**
Amplitude	(-0.050-0.334)	(-0.211-0.028)	(-0.025-0.01)	(-0.673-0.856)	**(0.001-0.494)**
Heritabilities	0.296± 0.102	0.095±0.095	0.056 ± 0.059	0.077 ± 0.059	0.094±0.075
	(0.093-0.485)	(0.000-0.330)	(0.00-0.215)	(0.001-0.271)	(0.01-0.271)
Trait means	13.154 ± 0.138	3.692 ± 0.073	2.262 ± 0.025	3.895 ± 0.168	2.178 ± 0.115
	(12.891-13.43)	(3.550-3.835)	(2.212-2.309)	(3.567 -4.224)	(1.953-2.410)
C.biguttulus	Syllable Duration	Strophe Duration	Dominant Frequency	Onset Accentuation	Maximum Amplitude
Syllable Duration	**0.283±0.068**	0.264±0.133	-0.181 ± 0.202	0.049 ± 0.161	0.208 ± 0.194
	(0.163-0.421)	(0.014-0.526)	(-0.551-0.224)	(-0.242-0.394)	(-0.216-0.534)
Strophe Duration	0.038±0.018	**0.076±0.014**	-0.078 ± 0.208	0.429±0.139	-0.652±0.117
	(0.002-0.072)	**(0.047-0.10)**	(-0.503-0.308)	(0.168-0.704)	(-0.838- -0.371)
Dominant Frequency	-0.006 ± 0.007	-0.001 ± 0.003	**0.004±0.002**	-0.142 ± 0.212	-0.054± 0.245
	(-0.019-0.008)	(-0.008-0.005)	**(0.001-0.009)**	(-0.520-0.285)	(-0.518-0.45)
Onset Accentuation	0.012 ± 0.05	0.072±0.024	-0.006±0.009	**0.391±0.116**	-0.376± 0.177
	(-0.089-0.108)	(0.027-0.119)	(-0.026-0.009)	**(0.185-0.639)**	(-0.696--0.011)
Maximum Amplitude	0.049±0.048	-0.069 ± 0.024	-0.002 ± 0.006	-0.376 ± 0.177	**0.15 ± 0.06**
	(-0.029-0.15)	(-0.111--0.025)	(-0.015-0.01)	(-0.696--0.011)	**(0.053-0.286)**
Heritabilities	0.499 ± 0.095	0.542 ± 0.076	0.127 ± 0.061	0.157±0.048	0.258±0.093
	(0.318-0.666)	(0.364-0.651)	(0.025-0.264)	(0.073-0.257)	(0.096-0.449)
Trait means	5.813 ± 0.056	1.893 ± 0.032	2.011± 0.011	6.309 ± 0.227	1.864 ± 0.045
	(5.705-5.925)	(1.832-1.955)	(1.988-2.033)	(5.865 -6.769)	(1.776-1.953)

Table 2: Flury hierarchy model comparison for **G** matrices of the two species. AIC values were averaged across 2,000 MCMC samples and models are ranked by decreasing AIC value. CPC = Common principle component (with numbers showing the number of CPCs).

Model	**AIC**	**Loglik**	**ΔAIC**
Heterogeneity	30	-15	0.0 ± 0.0
CPC(1)	41.4	-19.7	12.5 ± 16.0
CPC(2)	55.6	-25.8	26.7 ± 23.2
CPC(3)	68.9	-31.5	40.0 ± 28.1
CPC	77.7	-34.8	48.8 ± 30.1
Proportionality	201.3	-95.7	172.4 ± 112.2
Equality	276	-128	247.1 ± 140.5

Table 3 Phenotypic variance covariance matrix for five song traits in *Gomphocerippus rufus* and *Chorthippus biguttulus.* Total phenotypic variance is shown in the diagonals (bold). The phenotypic covariances are in the lower off-diagonals, while the phenotypic correlations are in the upper off-diagonals. Original variances are shown along with SD of the traits. Point estimates are accompanied with ±SE, and upper and lower CI.

G.rufus	**Syllable Duration**	**Strophe Duration**	**Dominant Frequency**	**Onset Accentuation**	**Maximum Amplitude**
Syllable Duration	**1.793 ± 0.18 (1.480-2.177)**	0.443±0.118 (0.217-0.658)	0.521 ± 0.497 (-0.397-1.516)	0.097 ± 0.091 (-0.065-0.283)	0.101 ± 0.10 (-0.101-0.298)
Strophe Duration	0.233±0.069 (0.108-0.371)	**0.702±0.061 (0.594-0.833)**	0.248 ± 0.126 (-0.004-0.481)	0.16 ± 0.117 (-0.071-0.387)	-0.856 ±0.106 (-1.056--0.63)
Dominant Frequency	0.016 ± 0.015(-0.012-0.047)	0.017±0.009 (0.000-0.036)	**0.041±0.004 (0.034-0.052)**	-0.75 ± 0.633 (-1.957-0.506)	-1.03 ± 0.558 (-2.162-0.081)
Onset Accentuation	0.085 ± 0.081 (-0.059-0.252)	0.061 ± 0.045 (-0.030-0.148)	-0.017 ± 0.016 (-0.050-0.011)	**1.053±0.177 (0.821-1.498)**	-0.049 ±0.094 (-0.223-0.14)
Maximum Amplitude	0.097 ± 0.099 (-0.098-0.301)	-0.338 ± 0.064 (-0.470--0.226)	-0.023 ± 0.013 (-0.051-0.002)	-0.033 ± 0.067 (-0.163-0.10)	**1.692±0.133 (1.475-1.976)**
Original Variance [SD]	1.875 [1.369]	0.648 [0.805]	0.042 [0.205]	0.938 [0.969]	1.611 [1.269]

C.biguttulus	**Syllable Duration**	**Strophe Duration**	**Dominant Frequency**	**Onset Accentuation**	**Maximum Amplitude**
Syllable Duration	**0.563±0.038 (0.495-0.646)**	0.467 ±0.183 (0.119-0.814)	-0.579 ± 0.445 (-1.408-0.316)	-0.016 ± 0.037 (-0.093-0.057)	0.581 ± 0.091 (0.400-0.75)
Strophe Duration	0.031±0.013 (0.008-0.057)	**0.139±0.009 (0.122-0.157)**	-0.006 ± 0.176 (-0.365-0.348)	1.021±0.143 (0.733-1.286)	-1.897 ±0.114 (-2.106--1.658)
Dominant Frequency	-0.006 ± 0.004 (-0.014-0.003)	0.00 ± 0.002 (-0.005-0.005)	**0.034±0.001 (0.031-0.037)**	-1.391 ± 0.541 (-2.340--0.215)	-0.404 ±0.441 (-1.280-0.504)
Onset Accentuation	-0.015 ± 0.038 (-0.092-0.06)	0.135±0.026 (0.092-0.192)	-0.027 ± 0.011 (-0.045--0.005)	**2.518±0.365 (2.092-3.442)**	-0.337 ±0.078 (-0.487--0.186)
Maximum Amplitude	0.123±0.025 (0.079-0.176)	-0.113 ± 0.013 (-0.140--0.09)	-0.004 ± 0.004 (-0.012-0.004)	-0.139 ± 0.034 (-0.208--0.078)	**0.575±0.03 (0.522-0.642)**
Original Variance [SD]	0.479 [0.692]	0.114 [0.337]	0.031 [0.177]	2.061 [1.436]	0.521 [0.722]

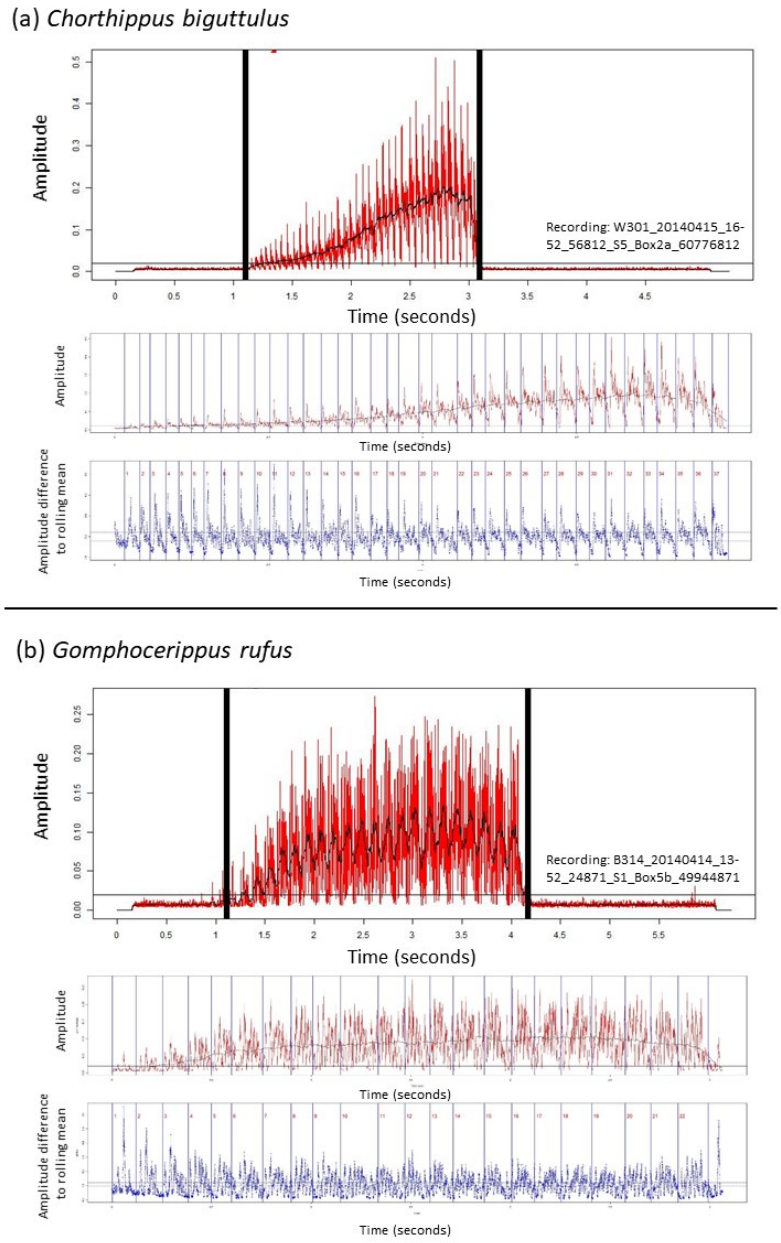

Figure 1: Examples of amplitude envelopes for strophes of (a) *Chorthippus biguttulus* and (b) *Gomphocerippus rufus*. The upper plot in each example shows the full strophe as it was recorded by Sound Analysis Pro with the boundaries (solid black vertical lines) of each strophe identified automatically using a custom R script. The black line shows the rolling average. The lower two plots show the syllable identification procedures with automatically identified syllables separated by vertical lines (and numbered in red on the lower plot). The upper of the two plots shows the absolute amplitude in red with the rolling average in black. The lower of the two plots shows the local deviation from the rolling average that facilities syllable identifications independent of fixed amplitude thresholds.

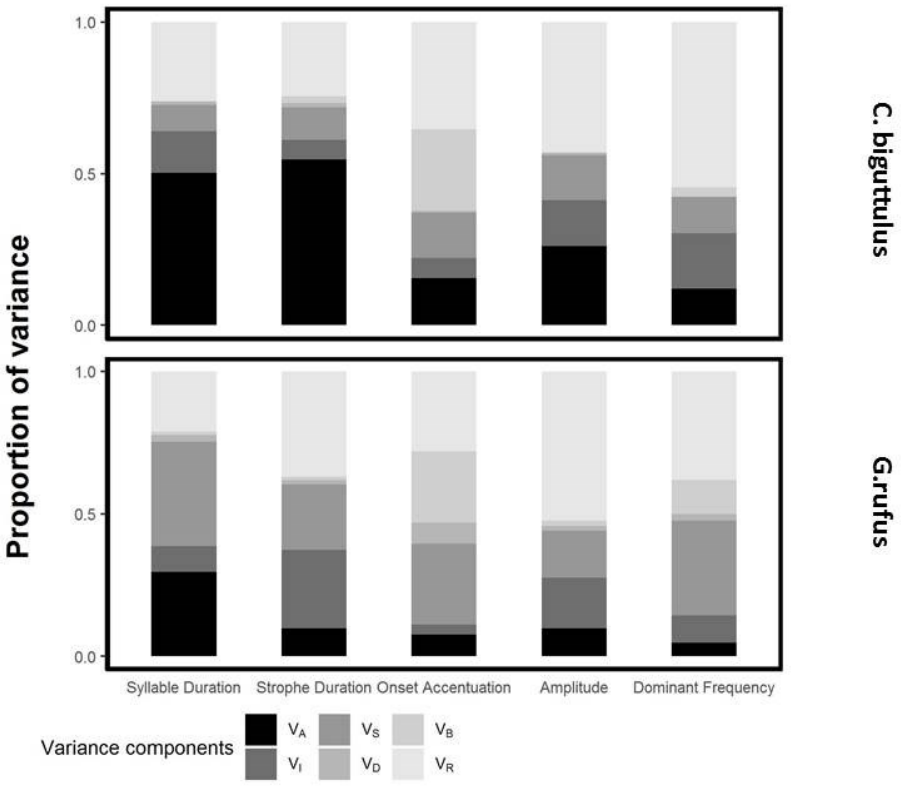

Figure 2: Variance components of five song traits in *C.biguttulus* and G.*rufus*. V_A is the additive genetic variance, V_I is the identity variance, V_S is the individual session variance, V_D is the day variance, V_B is the box variance and V_R is the residual variance.

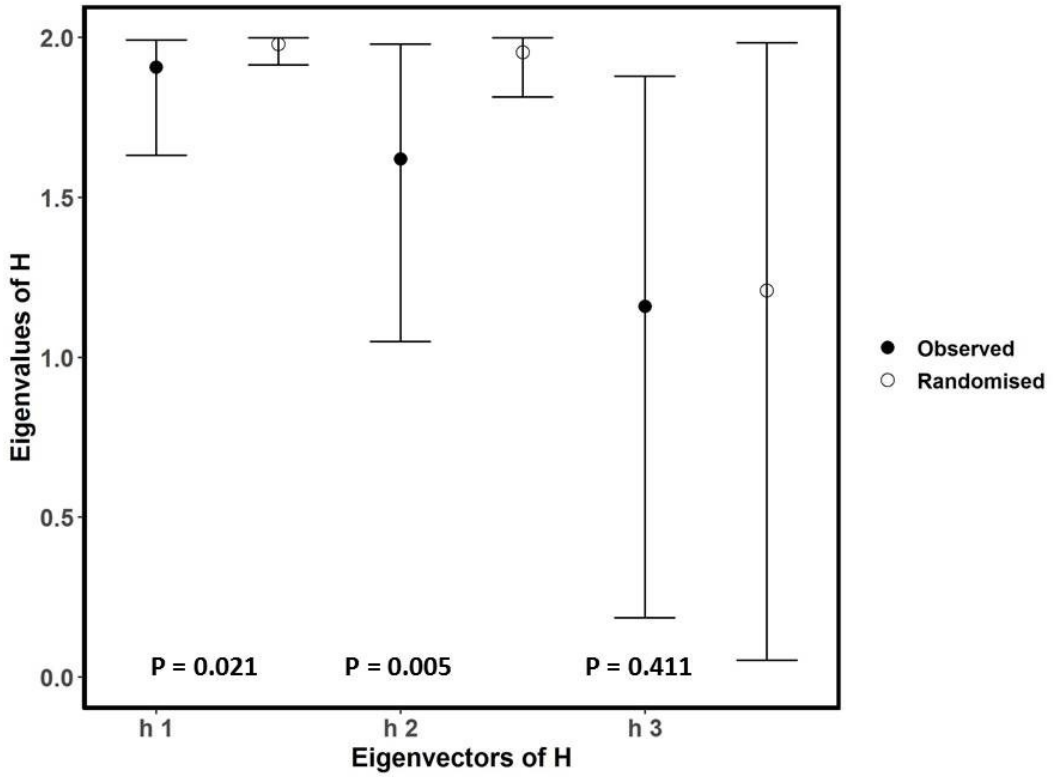

Figure 3: Krzanowski's subspace **H** for the comparison of **G** matrices among two species of grasshoppers. The x-axis denotes the three eigenvectors $\mathbf{h}_1$ to $\mathbf{h}_3$ of the **H** matrix and the y-axis denotes the eigenvalues of **H**. Filled symbols show empirical estimates with 95% CI and open symbols show randomized values. P values denote the proportion of randomized values that show equal or lower values than empirical estimates (incorporating variability in both empirical and randomized values).

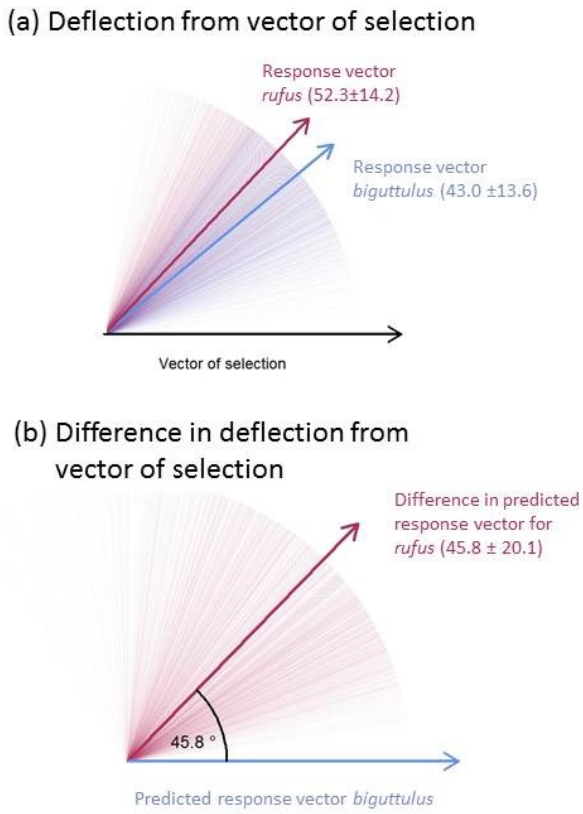

Figure 4: Random skewer projections through **G** matrices. a) Deflection angles between the vector of selection and response vectors based on 2,000 random skewer projections per species, b) Angle of deflection between response vectors of *C. biguttulus* and *G. rufus*. Thin lines show individual deflection angles, while the mean angle is shown in bold for each species (blue for *C biguttulus*, maroon for *G rufus*). Estimates show mean ± SE (95% CI) for the angles

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RESEARCH PAPER

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Direct and indirect genetic effects on reproductive investment in a grasshopper

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Abstract

A fundamental part of the quantitative genetic theory deals with the partitioning of the phenotypic variance into additive genetic and environmental components. During interaction with conspecifics, the interaction partner becomes a part of the environment from the perspective of the focal individual. If the interaction effects have a genetic basis, they are called indirect genetic effects (IGEs) and can evolve along with direct genetic effects. Sexual reproduction is a classic context where potential conflict between males and females can arise from trade-offs between current and future investments. We studied five female fecundity traits, egg length and number, egg pod length and number and latency to first egg pod, and estimated the direct and IGEs using a half-sib breeding design in the grasshopper *Chorthippus biguttulus*. We found that the male IGEs were an order of magnitude lower than the direct genetic effects and were not significantly different from zero. However, there was some indication that IGEs were larger shortly after mating, consistent with the idea that IGEs fade with time after interaction. Female direct heritabilities were moderate to low. Simulation shows that the variance component estimates can appear larger with less data, calling for care when interpreting variance components estimated with low power. Our results illustrate that the contribution of male IGEs is overall low on the phenotypic variance of female fecundity traits. Thus, even in the relevant context of sexual conflict, the influence of male IGEs on the evolutionary trajectory of female reproductive traits is likely to be small.

KEYWORDS

Acrididae, female fecundity, heritability, interacting phenotypes, Orthoptera, post-copulatory sexual selection, quantitative genetics, sexual conflict

1 | INTRODUCTION

Phenotypes evolve when selection acts on heritable trait variation. In order to understand speed and trajectory of adaptive evolution, we need to know the strength of selection and the degree to which phenotypic variation is inherited between generations (Falconer & Mackay, 1996; Lynch & Walsh, 1998). The field of quantitative genetics has therefore been concerned with estimating the additive genetic variances of traits under selection, largely treating all external influences as environmental without much differentiation

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(Falconer & Mackay, 1996; Lynch & Walsh, 1998). However, inheritance is sometimes more convoluted. Trait expression can, for example, be influenced by other individuals, that, from the perspective of the focal individual, represent part of the environment (Cheverud & Moore, 1994). If parts of the interactive influence have a heritable basis, then the interaction effect itself can evolve (Bijma, 2014; Cheverud & Moore, 1994; Kirkpatrick & Lande, 1989). Such heritable influences of the interacting conspecific represent indirect genetic effects (IGEs) from the perspective of the focal individual and can coevolve with direct genetic effects (Moore, Brodie, & Wolf, 1997).

Indirect genetic effects can be pivotal in the evolution of complex phenotypes as they can affect the response to selection. Without IGEs, the response to selection *R*, that is the change in trait means between generations, is determined by the heritability h^2 and the strength of selection represented by the selection differential *S*, as summarized by the breeder's equation $R = h^2S$. When a trait is influenced by both direct and indirect genetic effects, the magnitude and direction of the response to selection may deviate from this simple prediction (Agrawal, Brodie, & Wade, 2001; Kirkpatrick & Lande, 1989; Marie-Orleach et al., 2017; Miller & Moore, 2007; Petfield, Chenoweth, Rundle, & Blows, 2005; Wolf, Brodie, Cheverud, Moore, & Wade, 1998). In particular, IGEs can change the covariance between the additive genetic value and the phenotypic value of an individual and can therefore affect the entire dynamics of evolution, which can give rise to unintuitive scenarios (Arnold, 1994; Wolf et al., 1998). A negative genetic covariance between direct and indirect genetic effects can possibly lead to an evolutionary constraint which slows down the response to selection of the interacting trait even when the trait is positively selected (Wilson et al., 2011). Hence, IGEs can affect phenotypic evolution in intricate and subtle ways.

Indirect genetic effects can play substantial role in the evolution of group living with repeated interactions and cooperation (Alemu, Berg, Janss, & Bijma, 2014; Linksvayer & Wade, 2005; Wilson, Gelin, Perron, & Reale, 2009), since performance of the group (foraging success, protection from predators, aggressive interactions within the group) is partly influenced by the genetic background of individual group members. Consequently, IGEs have become important in applied animal breeding where multi-level selection at the level of the group and at the level of the individuals improves yields (Bijma, Muir, Ellen, Wolf, & Van Arendonk, 2007; Bijma, Muir, & Van Arendonk, 2007). In addition, parental interaction with offspring provides scope for IGEs mostly in form of maternal and paternal effects (Cheverud & Moore, 1994; García-González & Simmons, 2007). Similarly, interactions among siblings are inclined to involve a component of IGE.

Another intriguing facet of IGEs is their potential role in male–female interactions during mating by manipulating reproductive traits of the other mating partner (Filice & Long, 2017; Marie-Orleach et al., 2017; Petfield et al., 2005). IGEs may affect members of a pair bond, for example if females differentially allocate resources into reproduction depending on the attractiveness of the mating partner (Bolund, Schielzeth, & Forstmeier, 2009; Burley, 1988; Horváthová, Nakagawa, & Uller, 2011) or if sexual conflict gives rise to IGEs (Moore & Pizzari, 2005). Life history traits like laying date in birds, a trait that can be subject to sexually antagonistic selection, can also be influenced by male IGE (Brommer & Rattiste, 2008). IGEs are not restricted to animal interactions and can shape heritable variation in nearby conspecific plants (Costa e Silva, Potts, Gilmour, & Kerr, 2017). Overall, IGEs are likely to be more extensive in nature than commonly appreciated, but their magnitude and thus contribution to phenotypic evolution may be context dependent.

Indirect genetic effects can be estimated using two complementary approaches (McGlothlin & Brodie, 2009). Frequently, IGEs are estimated using the interaction coefficient ψ (Moore et al., 1997). This requires that the trait that influences the phenotype of the interaction partner is known and measurable, a condition that will often not be fulfilled. Alternatively, IGEs can be quantified using variance decomposition just like in classical quantitative genetics where the pedigree relationships of both interacting partners are fitted to predict the phenotype of the focal individual (Arango, Misztal, Tsuruta, Culbertson, & Herring, 2005; Bijma, 2010). This approach works even when the influential phenotypes are unknown or unmeasurable. The methodological framework of the animal model for estimating IGEs via variance decomposition is well established (Kruuk, 2004). Moreover, IGEs are outcome of complex scenarios. So there can be a suite of traits of the interaction partner, rather than a single trait which might drive the IGEs. In that case, considering a single trait would produce biased results (Prokop & Drobniak, 2016). The variance decomposition approach integrates all these unknown traits of the interaction partner and gives a more comprehensive picture of the total contribution of IGEs.

We here study interacting phenotypes in a mating context. IGEs are particularly likely to evolve in this context, because of the scope for conflict of interest between the sexes (Arnqvist & Rowe, 2005; Parker, 1979). Indeed, variance component approaches have shown IGEs to affect timing of reproduction in common and red-billed gulls (Brommer & Rattiste, 2008; Teplitsky, Mills, Yarrall, & Merilä, 2010). Since reproductive performance is closely linked to fitness, direct as well as indirect genetic effects are likely to be under selection in both males and females and evolve jointly, potentially leading to escalated conflicts (Holland & Rice, 1998). The evolutionary interests of males and females are particularly disparate in species in which multiple mating occurs regularly, because males and females often have different strategies to maximize their fitness and exploit each other (Moore & Pizzari, 2005; Rice, 1996). Besides conflict over re-mating rates (Arnqvist & Rowe, 2005; Bateman, 1948), there can be conflict over the trade-off between current and future reproduction (Williams, 1966). While females will be selected to hold back some resources for future reproduction, it is the evolutionary interest of males that their partners invest as much as possible and as soon as possible, because paternity for later reproductive events is likely to be lost to other males.

Sexual conflict is common among many insects that are polygamous and iteroparous (Arnqvist & Rowe, 2005) and can take the form of conflict in terms of timing of reproduction and resource allocation (Arnqvist & Nilsson, 2000). There is also a physiological basis for how male insects can potentially manipulate female fecundity post-copulation, since seminal fluid or contents of the spermatophore can have physiological effects on females (Hartmann & Loher,

1996, 1999). The scope for conflict and the existence of a potential mechanism for manipulation make fecundity traits particularly prone to be influenced by IGEs. At the same time, fecundity traits are directly related to life-time reproductive success and their inheritance patterns are thus obviously relevant to evolutionary trajectories. Fecundity traits are also interesting, because they are exclusively expressed by females and yet can be indirectly affected by males.

Here, we address the contribution of male indirect genetic effects to phenotypic variation in female reproductive traits of the bow-winged grasshopper, *Chorthippus biguttulus*. Every few days, females lay an egg pod consisting of typically 6–10 eggs. There is no extended mate guarding, but males can potentially affect female reproductive traits via some post-copulatory effects, including seminal fluid proteins and amount of sperm (Gregory, 1965; Zhu & Tanaka, 2002). Indeed, substances in the spermatophore of grasshoppers are known to influence re-mating behaviour of females for some days following mating (Hartmann & Loher, 1996, 1999). We expect manipulative male IGEs to increase the number of eggs, size of egg pods and the number of egg pods shortly after mating. While females are selected to balance current with future reproduction, this is not the case for males that are likely to lose paternity of future offspring to later mating partners. We therefore predict that males are selected to manipulate their mating partners to lay eggs sooner, more in number and larger with more resources, leading to a scenario of plausible sexually antagonistic coevolution (Chapman, Arnqvist, Bangham, & Rowe, 2003; Moore & Pizzari, 2005).

We used a half-sib breeding design to disentangle the direct and indirect genetic effects on five female fecundity traits (latency to first egg pod, egg pod length and number, egg length and number). Egg pods represent clutches of a few eggs packaged in sand and foamy substance to form a case. The foam is secreted from the female accessory glands and when dried acts as an internal support for the eggs (Waloff, 1950) and also acts as an anti-microbial agent (Chapman & Joern, 1990). All traits were thus chosen for being close to evolutionary fitness and at the same time potentially subject to conflict of interest between the sexes as laid out above. We estimated the (direct) heritability along with the contribution of male IGE to the phenotypic variance of these female-limited reproductive traits using an animal model-based variance decomposition approach. We consider these traits to be particularly promising for the evolution of IGE, because of the sexual conflict situation and also because of the potential mechanistic basis of IGE via the physiological effects of the spermatophore that males transfer to females. The contribution of IGEs to female fecundity traits is currently unknown for this species and also for iteroparous, species without pair bond in general.

2 | MATERIALS AND METHODS

2.1 | Source population and housing

We used the bow-winged grasshoppers, *C. biguttulus* (Acrididae, Orthoptera), as our study organism. The bow-winged grasshopper is a common species in open terrestrial habitats throughout large parts of Eurasia. We collected last instar nymphae from the field. After final moult into the reproductive imago stage, individuals were separated by sex and kept temporarily in mesh cages of dimension 22 × 16 × 16 cm with up to three individuals per cage. Freshly cut grass was provided in small vials filled with water and was replaced with fresh grass when it started to wilt.

2.2 | Breeding design

We set up a half-sib breeding design to unravel the role of direct and indirect genetic effects on reproductive traits of females. Females were separated in individual cages (22 × 16 × 16 cm) with sand pots provided as egg-laying substrate. A total of 73 males were mated to two virgin females each. Males were swapped between cages of their assigned females every few days until males died. Females that died before they had laid eggs were replaced by new virgin females. Egg pods were collected from the sand pots once per week and were from thereon kept in Petri dishes lined with filter paper. The filter paper was sprayed with water once per week to keep eggs moist. After about six weeks at room temperature, eggs pods were transferred to refrigerators where they were kept at low above-zero temperature from October till they were taken out for hatching.

2.3 | F1 generation

Eggs were taken out of their simulated winter conditions in five cohorts between January and June. Hatchlings appeared after about two weeks at room temperature. All hatchlings from the same egg pod were transferred to separate family cages (22 × 16 × 16 cm) where they were housed together until they matured. This procedure allowed us to trace ancestry. It is impossible to persistently mark individuals across the four juvenile moults, which precluded raising subjects in groups of mixed origin. However, most females laid multiple egg pods and their resulting offspring were raised separately. The population was maintained at a room temperature of 25–30°C and a relative humidity of 35%–55%. Fresh food was provided as in the parental generation. On the day of moulting into imagoes, individuals were removed from their family cages. Females were transferred to individual housing cages (22 × 16 × 16 cm) with fresh grass for food and sand pots as egg-laying substrate. Each female received its separate cage. Males were individually marked with bee tags and were temporarily transferred to communal cages (47.5 × 47.5 × 93 cm) with about 25 individuals in each cage.

2.4 | Mating and reproduction

On the day females reached an imaginal age of 7 days, and a single, randomly selected mature male was transferred to each female cage. Usually, grasshoppers exhibit a primary defence against copulation attempts between 3–5 days after emergence as adults (Hartmann & Loher, 1974), so we used females that were slightly beyond this age

and thus willing to mate (see also Wirmer, Faustmann, & Heinrich, 2010). Females did not have any previous contact with reproductive males and thus were virgin. Observations showed that males typically displayed to females shortly after being introduced to the female cage, and many successful copulations were observed within the first few hours. After a full day in the female cage, the male was removed from the cage and females were subsequently housed without mating partners, but still with egg-laying substrate. Egg pods were collected on a daily schedule until day 15 after mating. Egg pods were kept in labelled Eppendorf tubes until they were dissected for further measurements.

2.5 | Morphometrics

We measured the length of egg pods using a digital slide caliper with a Vernier scale to the nearest 0.001 mm. Egg pods were then dissected under a stereo microscope, and the number of eggs was recorded. We took standardized photographs of all eggs from every egg pod. Egg length was measured from images using the ImageJ 1.46r software (Schneider, Rasband, & Eliceiri, 2012). Along with the date of laying, these data gave us information on five reproductive phenotypes of females: latency to first egg pod, number of egg pods, egg pod length, number of eggs per egg pod and egg length. Eggs from a subset of 77 egg pods (912 eggs in total) were measured twice from the same image, and the repeatability was estimated using the rptR package in R (Stoffel, Nakagawa, & Schielzeth, 2017).

2.6 | Sample sizes

Altogether we recorded the fecundity of 412 females. These females originated from 280 distinct egg pods from 93 distinct mothers and 64 distinct fathers. They had been mated to 361 males that originated from 299 distinct egg pods from 94 distinct mothers and 68 distinct fathers. Most males were used once, but around 16% of the males were mated more than once. We collected 1,062 eggs pods from focal females containing a total of 6,906 eggs.

2.7 | Variance decomposition

Our aim was to untangle the direct genetic effects of females and the indirect genetic effects of the males on the focal female's reproductive traits. We used R 3.4.0 (R Core Team, 2017) to fit all models. We fitted animal models using the package pedigreemm (Vazquez, Bates, Rosa, Gianola, & Weigel, 2010), which is an animal model extension to the popular lme4 package, and with a Bayesian approach as implemented in the MCMCglmm package (Hadfield, 2010). Models fitted in pedigreemm were used for significance testing while MCMCglmm models were used for estimation. Number of eggs per pod, egg pod length and egg length were modelled with Gaussian error distributions while latency to first egg pod, and number of egg pods were modelled using Poisson distributions with log link. There were seven records of unusually large numbers of eggs per pod (up to 19, see Results section) that were winsorized to 12 in order to mitigate the effect of these outliers. Results are qualitatively similar with and without this procedure.

We performed separate univariate animal model analyses for all four fecundity traits (except latency to first egg pod) separated by 15 different time intervals along the 15 days post-mating period. For latency to first egg pod, a trait that is entirely determined by the temporal sequence, we did not form subsets and analysed the full data in a single model. The separation by time points was introduced in order to accommodate the potential trade-off between effect size and statistical power: The magnitude of indirect genetic effects potentially fades with time after mating while power for detecting indirect genetic effects may increase with the accumulation of data over the 15 days period after mating. We therefore generated 15 subsets of the data for each trait, starting with all data collected up to day 1 after mating through to all data collected up to day 15. This is equivalent to an incremental increase of data in steps of 1 day and each subset thus contained all data from the previous subset. For the number of egg pods, we included all individuals in each subset, with a score of zero for all individuals that had not laid eggs up to the specified day. In addition to this analysis, we also fitted models with data limited to specific dates (without incremental increase of data). Results are presented in the Supporting information Figure S11 and S12.

2.8 | Model structure

The basic structure of a univariate model was as follows:

$$\mathbf{y}=\mathbf{X}\boldsymbol{\beta}+\mathbf{Z}_1\mathbf{f}+\mathbf{Z}_2\mathbf{m}+\mathbf{Z}_3\mathbf{c}+\mathbf{Z}_4\mathbf{p}+\mathbf{e} \quad (1)$$

where $\mathbf{y}$ is the response vector, $\boldsymbol{\beta}$ is the vector of fixed effects estimates, $\mathbf{f}$ is the vector of female additive genetic effects (female identity linked to the pedigree), $\mathbf{m}$ is the vector of the male indirect genetic effects (male identity linked to the pedigree), $\mathbf{c}$ is the vector for cohort effects (cohort identity), $\mathbf{p}$ is the vector of pair identity effects (that control for repeated phenotypic measurements resulting from a single mating), $\mathbf{e}$ is the vector of residuals, $\mathbf{X}$ is the design matrix for the fixed effect and $\mathbf{Z}_1$, $\mathbf{Z}_2$, $\mathbf{Z}_3$, $\mathbf{Z}_4$ are the incidence matrices of the respective random effects. The pair identity effect in our design captures female nongenetic and permanent environment effects, male nongenetic and permanent environment effects as well as male–female genetic and environmental interaction effects (see also Discussion section). Since we were phenotyping F1 offspring, part of the pedigree variance might arise from parental effects, even though other parts of the parental effects will also be captured by the pair identity effect. As fixed effects, we fitted the time difference between mating and laying date to control for temporal trends in fecundity traits.

Some adjustments to the basic model had to be made for different traits (Supporting information Table S2). When modelling egg length, we added egg pod identity as an additional random effect ($\mathbf{Z}_5\mathbf{ep}$), which accounted for the variance in egg length between egg pods. For the traits which were modelled by Poisson distribution (number of egg pods and time to first egg pod), we added

an observation-level random effect to model overdispersion. We also omitted pair identity and days after mating from these two traits, since these traits were not replicated within pairs. As a consequence, indirect and direct genetic components might be more strongly inflated by parental effects for these traits. When modelling the number of egg pods, we controlled for whether the female died or not as a categorical fixed effect as well as age of death in days after mating as a continuous covariate (values set to 16 for those who did not die during the experimental period) to control for the fact that dying females are expected to lay fewer egg pods. The strategy to fit a categorical predictor (died yes/no) and a continuous predictor allows the model to estimate the regression slope for date of death based on nonsurvivors while allowing survivors to assume any value without being constrained by the regression line for nonsurvivors.

2.9 | Model fitting and estimation

For models fitted in MCMCglmm, we used dispersed default priors for fixed effects and parameter expanded (half-Cauchy) priors for the random effects (Gelman, 2006). The degree of belief parameter ν for the random effects was set to 1 and that of the residuals was set to 0.002. The posterior distribution of the models was obtained from 110,000 iterations with a thinning interval of 100 and a burnin of 10,000 for all the Gaussian traits, while that of the Poisson distributed traits were estimated from 1,100,000 iterations with a thinning interval of 1,000 and a burnin of 100,000. Model convergence was visually inspected from the trace plots and also using Gelman and Rubin diagnostics (Gelman & Rubin, 1992). The cohort random effect with few group-level replicates (five cohorts) occasionally produced large estimates in some iterations, but this had little effect on the estimated ratios.

We used likelihood ratio tests for significance testing by comparing the difference in likelihoods between the full model and a reduced model with the focal random effect removed, one at a time, against a mixture distribution between X^2_0 and X^2_1 because variances were tested at the boundary of possible parameter space [i.e., against zero variance (Self & Liang, 1987)]. As some of the models did not converge in pedigreemm, the log likelihoods must be treated with caution. For the uncertainty of estimates, we present the posterior means of the estimates extracted from the MCMCglmm models along with the 2.5% and 97.5% quantiles (equivalent to confidence intervals) and standard deviations of the posterior distributions (equivalent to standard errors).

Variances were converted to proportion of the phenotypic variance explained by division with the sum of all random effect variances. Specifically, this converted direct and indirect genetic variances into heritabilities noting that these might be inflated by parental effects as discussed above. The heritabilities of Poisson traits were quantified on the link scale and on the data scale using the GLMM approach introduced by (de Villemereuil, Schielzeth, Nakagawa, & Morrisey, 2016) and implemented by the QGglmm R package. This approach uses integration over breeding values and fixed effects to quantify the amount of heritable variation that acts additively on the data scale. For comparison, we also present the distribution-specific variances as described for estimates of repeatabilities (Nakagawa & Schielzeth, 2010), but note that not all of the repeatability-based heritability is strictly additive on the data scale (see de Villemereuil et al., 2016).

2.10 | Randomization runs

With insufficient data for estimating variance components (due to low sample size, poor data structure or confounding of different variance components), posterior distributions broaden and the posterior mean tends to shift away from the boundary. This is potentially problematic, since we compare heritabilities when estimated with less data (shortly after mating) to those estimated with more data (including all data up to 15 days after mating). In order to verify that an increased posterior mean at the beginning of the post-mating period is not simply a statistical artefact, we performed a randomization procedure that generated null expectations. We therefore generated 100 randomized data sets, in which all trait-predictor links were kept intact except for the male pedigree links, for which we randomized male identity labels. The randomization of male identity labels ensured that repeated observations from the same male were kept together and that family sizes and the number of male pedigree links were kept identical across randomizations. Only the association between pedigree and phenotypes was broken. For each randomized data, we refitted the model and extracted the posterior distribution of the male pedigree effect. Since data are successively added (from a 1-day to a 15-day threshold) and are thus partially redundant, we limited the randomization procedure on five representative time points (days 1, 3, 5, 10 and 15).

3 | RESULTS

We measured the length of 6,413 eggs in total with a repeatability of $R = 0.910 \pm 0.008$ *SE* in a subset of 464 eggs from 78 egg pods that were measured twice independently. The mean (*SE*) egg length at the start of the experiment was 3.50 ± 0.10 mm on the day following mating and increased per week by 0.042 ± 0.002 mm on average (Figure 1a). Across the full 15-day observation period, variation in egg length was explained to roughly equal amounts by direct genetic effects (20%), shared environmental cohort effects (20%), pair interaction effect (10%) and family effects shared among eggs from the same egg pod (15%) (last column in Figure 2a). The estimate of indirect genetic effect was almost an order of magnitude smaller than the other sources of variance (1%) and not significantly different from zero (Table 1). When considering the first few days only, the male indirect genetic effect was estimated somewhat larger (7.6% on day 1), but this might be primarily an effect of little data and was not statistically significant (Supporting information Figure S1). Already after about 6 days of data collection, the estimates remained remarkably constant even with the addition of further data

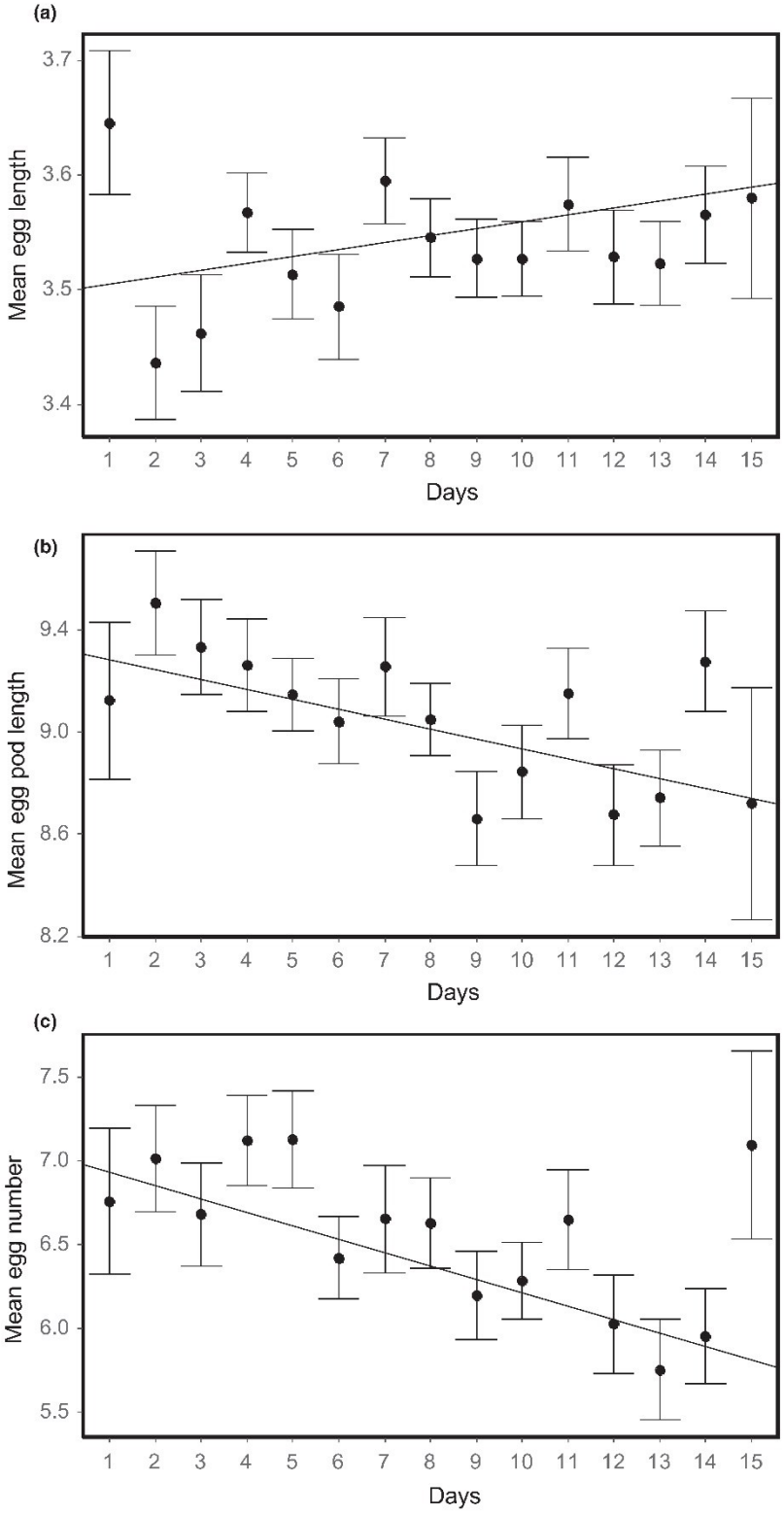

FIGURE 1 Mean trait values of (a) egg length, (b) egg pod length and (c) number of eggs per pod plotted against time after mating. Means and SEs were extracted from mixed models fitted only to data for the specified date. The regression lines are estimated from the slopes and intercepts extracted from an animal model fitted to the full data set

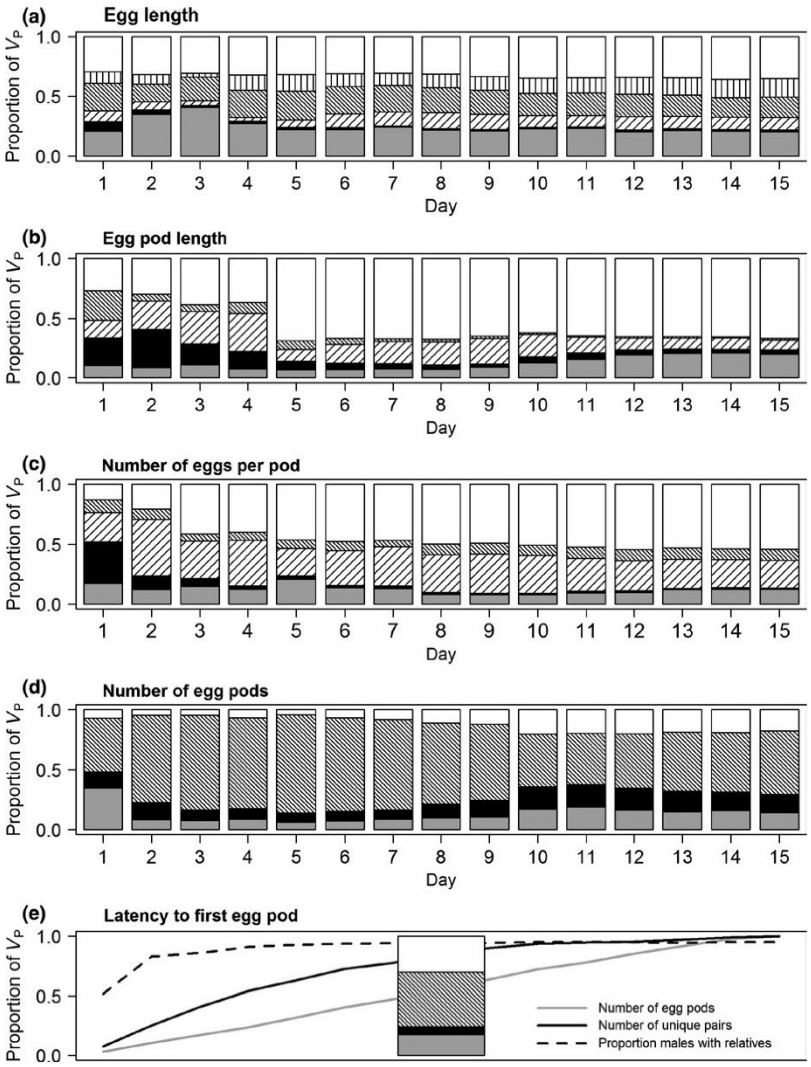

FIGURE 2 Variance components for (a) egg length, (b) egg pod length, (c) number of eggs per pod, (d) number of egg pods and (e) latency to first egg pod across the 15-day period estimated separately across 15 data sets that include successively more data up to and including the specified day (see Methods section). Female additive genetic effects are shown in solid grey, male indirect genetic effects are show in solid black, pair identity effects in coarse diagonal hatching, the cohort effect in dense diagonal hatching, the egg identity effect in coarse vertical hatching and the residual variance component in white. Variances of Poisson traits are shown on the link scale. For latency to first egg pod, there is no incremental increase in the amount of data and the bars refer to the full 15-day period. The lines in plot (e) show the cumulative increase in the amount of data across the 15-day period for the number of egg pods (solid grey line), the number of unique males that contribute to the data (solid black line) and the proportion of males that are linked to other males via the pedigree (dashed black line)

(Figure 2a). Posterior distributions of female direct genetic effect for all the data subsets on egg length and subsequent traits are shown in Figures S6–S10.

The mean egg pod length was 9.285 ± 0.159 mm on the day following mating and decreased by −0.273 ± 0.012 mm per week in parallel to the decrease in egg number (see below), but opposite to the increase in average egg length (Figure 1b). Across the full 15-day observation period, the cohort effect was very small (1.6%), the effect of pair identity was low (8%) and the direct heritability was moderately high (20%), with only the heritability being statistically significant (Table 1, Figure 2b). Indirect genetic effects of males were estimated small (3%) and not significantly different from zero (Table 1). When considering only the first few days, the male indirect genetic effect was estimated larger (Figure 2b), with 18%–14% estimated between days 1 and 4, and randomization suggests that this increase is larger than expected by chance (Supporting information Figure S2). This is consistent with the idea that IGEs are strongest shortly after an interaction.

The mean number of eggs per pod was 6.933 ± 0.456 on the day following mating with a decline by −0.560 ± 0.016 eggs per week (Figure 1c). Hence, at the end of the experiment, egg pods were smaller by one egg on average. Most egg pods contained 4–10 eggs with only 1% of all pods containing 11–12 eggs (Figure 3). Rather surprisingly, we found seven egg pods (0.7%) with more than 12 (max 19) eggs (Supporting information Table S1). Across the full 15-day observation period, variation in egg number was explained about equally by direct genetic effects (12%) and shared environmental cohort effects (12%) and even more by the pair interaction effect (22%), with large residual variation (Figure 2c). Indirect genetic effects of males were marginal in magnitude (1%) and not significantly different from zero (Table 1, Figure 2). Shortly after mating, the male indirect genetic effect was estimated larger (Figure 2c), 30% on day 1 but randomization suggest that these estimates are not larger than expected by chance (Supporting information Figure S3). Other variance components remain remarkably constant across all subsets of the data (Figure 2c).

TABLE 1 Estimates of variance components along with standard error, credible intervals and likelihood ratio test for the full 15-day period. As the model for latency to egg pod did not converge in REML, we could not test significance of the respective variance components. The Poisson variance was calculated following (Nakagawa & Schielzeth, 2010) with λ estimated from the mean of the raw data

Trait	Female (pedigree)	Male (pedigree)	Cohort	Mating pair identity	Egg pod identity	Residual	Poisson variance
Egg length	0.043 ± 0.014 (0.020–0.073) $\chi^2 = 27.75, p < 0.001$	0.003 ± 0.003 (0.000–0.012) $\chi^2 = 0.34, p = 0.56$	0.043 ± 0.081 (0.005–0.227) $\chi^2 = 24.97, p < 0.001$	0.022 ± 0.010 (0.001–0.042) $\chi^2 = 5.83, p = 0.015$	0.033 ± 0.003 (0.028–0.039) $\chi^2 = 492.70, p < 0.001$	0.074 ± 0.002 (0.071–0.077)	
Egg pod length	0.542 ± 0.181 (0.225–0.929) $\chi^2 = 17.9, p < 0.001$	0.088 ± 0.093 (0.000–0.329) $\chi^2 = 0.20, p = 0.65$	0.042 ± 0.273 (0.000–0.238) $\chi^2 = 0.0, p = 1.0$	0.223 ± 0.159 (0.002–0.577) $\chi^2 = 2.60, p = 0.10$	-	1.766 ± 0.102 (1.584–1.985)	
Egg number	0.783 ± 0.386 (0.154–1.713) $\chi^2 = 8.93, p = 0.003$	0.071 ± 0.097 (0.000–0.351) $\chi^2 = 0.0, p = 1.0$	0.836 ± 3.498 (0.057–3.782) $\chi^2 = 11.67, p < 0.001$	1.449 ± 0.384 (0.703–2.222) $\chi^2 = 16.06$, $p < 0.001$	-	3.410 ± 0.188 (3.050–3.790)	
Latency to first egg pod	0.056 ± 0.043 (0.000–0.163)	0.022 ± 0.022 (0.000–0.079)	0.374 ± 1.979 (0.025–1.926)			0.094 ± 0.046 (0.005–0.179)	0.18
Egg pod number	0.004 ± 0.005 (0.000–0.018) $\chi^2 = 1.09, p = 0.29$	0.004 ± 0.005 (0.000–0.018) $\chi^2 = 0.0, p = 1.0$	0.029 ± 0.083 (0.000–0.158) $\chi^2 = 0.0, p = 1.0$			0.004 ± 0.004 (0.000–0.013)	0.33

During the 15-day experimental period, females produced 2.50 ± 0.070 egg pods on average (considering only the 396 females that survived the full 15-day period). The variance components affecting egg pod number as estimated on the link scale across the full 15-day period were all small, except for the cohort effect that was very large (70%) (Figure 2d and Figure S4). When estimating the additive genetic components on the data-scale, the direct heritability was 1% and the estimate for male indirect genetic effect was 1%. Repeatability-based estimates were nearly identical to integration-based estimates of heritabilities on the data scale (Table 1). Unlike for other traits, the indirect genetic effect tended to increase with time (Figure 2d and Figure S4).

Latency to first egg pod was 5.040 ± 0.160 days after mating. The cohort effect was large (68%) and the direct heritability small (10%). Indirect genetic effects of males were estimated low (4%) and not significantly different from zero (Figure S5). On data scale, the direct heritability was 7% and the male effect was 3% (Figure 2e). Repeatability-based estimates were nearly identical to integration-based estimates of heritabilities on the data scale (Table 1). Due to the lack of replication at the level of individuals, the genetic components might be more strongly influenced by parental effects for latency and number of egg pods.

4 | DISCUSSION

We studied the direct and indirect genetic variance of reproductive traits in a situation of putative sexual conflict. Males are expected to have an evolutionary interest in manipulating their mating partners and, in grasshoppers, potentially have a mechanism for manipulation by physiologically active substances transferred with the spermatophore (Hartmann & Loher, 1996, 1999). We found the direct heritabilities of female fecundity traits to be moderate and the indirect genetic effects to be small and not statistically different from zero. However, indirect genetic effects were estimated larger a few days after mating, which may suggest that IGEs fade with time after mating. We found that egg length, pod length and egg number showed significant female additive genetic variance while this component was not statistically significant for latency to first pod and number of egg pods. This might be due to the necessarily less fine-grained measurements of the two Poisson traits, but might also reflect less additive genetic variance to timing and pattern of egg laying. It is important to remember that since the separation into genetic and parental components is incomplete, what we call genetic components might include parental effect that either have direct or indirect effects on female fecundity.

Egg length is determined by the amount of resources put into the egg and more resources likely provide benefits to the developing offspring. The amount of resources put into each egg is potentially subject to conflict between mating partners because of the trade-offs between resource allocation to current versus future reproduction that has different optima for females and males. But egg size is possibly also influenced by the size of the female and the size of

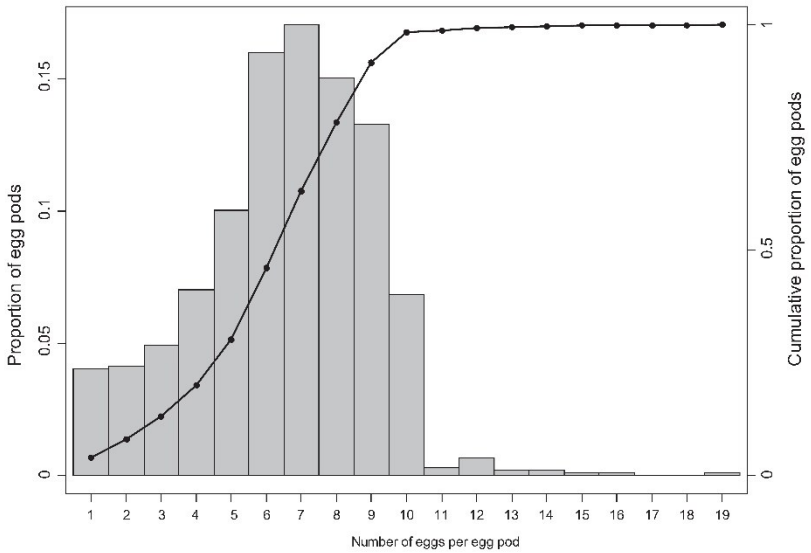

FIGURE 3 Frequencies (bars) and cumulative frequencies (line) of the number of eggs per egg pod

its body cavity which may limit the potential to which the egg size can be influenced by the mating partner. Interestingly, egg length increased steadily with the time after mating, whereas the egg number and pod length decreased. This might reflect an allocation trade-off which shifts with age and/or with the progress of the season. It is possible that some ovarioles become inactive with age, allowing the remaining ones to produce larger eggs, a possible physiological effect that may or may not be adaptive. The effect could be adaptive if eggs laid later in the season require more nutrients in order to develop sufficiently far before the onset of diapause. It might also be the case that because the females are getting sperm-depleted, they shift their investment into producing bigger eggs rather than increasing the number of eggs. It would be interesting to see in future experiments whether the trend changes when the females are re-mated.

The number of eggs per pod depends in part on the number of ovarioles present, which in the bow-winged grasshopper is 5–7 ovarioles per side, with a typical range of 1–10 eggs per pod (Table 1 in Ingrisch & Köhler, 1998). We found the full range of 1–10 eggs per egg pod at appreciable frequencies and a sharp decline thereafter (Figure 3). In grasshoppers, each ovariole can typically contribute only a single egg to each egg pod (Uvarov, 1966). Our data thus suggest that individuals in our population of the bow-winged grasshopper typically possess five pairs of ovarioles of which not all are used on every occasion. But there are also some indications for the presence of a polymorphism in the population as 0.7% of the females laid 11–12 eggs in a single egg pod. Very surprisingly, some of the females laid even more eggs in a single egg pod (Supporting information Table S1). All these pods were intact and thus did not arise from multiple pods glued together. There was neither any specific pattern of time when these eggs were laid nor was there any trend in the number of eggs present in other egg pods laid by the same female (Supporting information Table S1). We can only speculate that these females had an unusually large number of ovarioles or they produced multiple sets of eggs from the same ovarioles. The data are too scanty for firm conclusions, but the few cases where females laid large as well as normal egg pods (Supporting information Table S1) may suggest the latter option, since large egg pods are in some cases approximately twice the size of normal egg pods.

Egg number and egg length were significantly influenced by pair identity. The effect of pair identity pools several causal components in our experimental design. It captures the environmental (and nonadditive genetic) components to both the direct effect of the female and the indirect effect of the males. Additionally, it contains the interaction variance, that is the response of particular females to particular males, including the interaction of genetic variants in males and females. Such gene-by-gene interactions between interaction partners can sometimes be substantial (Rode, Soroye, Kassen, & Rundle, 2017) and do represent a part of IGEs that we have missed in our experimental design. However, while some of the pair identity effect may represent genuine G × G, we consider it likely that most of the effects is caused by female permanent environmental effects and assume that male–female interaction effects involving environmental components are still larger than G × G. For egg number and egg length, the effect of cohort was also significant which implied that systematic environmental influences are particularly relevant for those two traits.

Our results illustrate that under circumstances where IGEs are especially likely, because of the difference in evolutionary interests of the interaction partners, and where there is also a potential mechanistic basis for IGEs, their contribution to the phenotypic variances is apparently very small. While in most cases, we cannot distinguish whether IGEs are present in the sense of being statistically larger than zero, we can conclude that they are small and in most cases substantially lower than the direct genetic effects. Notable exception might be early effects on egg pod length and number of eggs per pod as well as the number of egg pods. Phenotypic evolution in female reproductive traits will thus be primarily driven by direct selection on female additive genetic

variance rather than indirectly via selection on male traits. It is worth to reconsider which IGEs we did cover in our experimental design. Males and females had interacted for 24 hr. The most likely physiological mechanism of manipulation is any physiological effect of substances transferred with the spermatophore (Hartmann & Loher, 1996, 1999). The demonstration that males manipulate female fecundity traits, however, does not ensure that there is also significant heritable variation in male manipulative traits. Our study shows that the contribution to the phenotypic variance in female fecundity is small, in any case. However, it is also possible that other aspects of the male phenotype may affect female fecundity traits. For example, females might respond to differences in male attractiveness, via differential resource allocation, for example by investing more resources when mated to an attractive male than when mated to an unattractive male (Burley, 1988). If attractiveness is heritable, such differential allocation may appear as an indirect genetic effect. Furthermore, it is conceivable that the developing embryo(s) may influence female reproductive investment as a form of early parent–offspring conflict. We would consider this an indirect genetic effect from the perspective of the female, although not via the phenotype of the male mating partner, but rather by its contribution to the offspring genotype. However, there is only a very short time span before egg-laying when zygote(s) can potentially influence female fecundity traits.

Overall, our results demonstrate low indirect genetic effects in a conflict scenario of female investment into reproduction after mating with a few possible exceptions. For egg pod length and number of eggs per pod, the IGE component was larger early after mating which is consistent with the idea that IGEs fade with time after interaction. However, our analyses also illustrate an interesting phenomenon of apparently larger variance components when less data is available, that may serve as a warning to future studies. Simulations illustrate that the increase in variance components is in most cases not larger than expected by chance. Furthermore, we report cases of unusually large numbers of eggs per egg pod that suggest that grasshoppers can sometimes encase multiple eggs from the same ovarioles into a single egg pod.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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General discussion

In this thesis I have explored two categories of questions — firstly, comparing the stability of additive genetic variance covariance matrix, **G**, for conserved morphological traits and for more variable song traits in grasshoppers, and secondly, estimating male indirect genetic effect and female direct genetic effect on female fecundity traits of a grasshopper under a potential sexual conflict scenario. Here I list the major results from the three thesis chapters. I discuss those results in detail in the following sections.

I found that the **G** matrices for the conserved morphological traits showed substantial differences and have diverged among species and the **G** matrix divergence follows a phylogenetic signal, i.e. more related species have more similar **G** matrix. The analyses identified wing length as the most influential trait which contributes considerably to matrix differences. The genetic constraints among the morphological traits were low to moderate, and the cross-sex correlation which is indicative of the potential of sexual dimorphism is also moderate.

1. In the second chapter of my thesis, I found the **G** matrices of male ornamental traits, i.e., song traits are not stable between the two species and have diverged. The song traits showed high to moderate heritabilities in the two species, but the heritabilities of traits which are previously known to be under selection are low. The genetic correlations are also higher in one species which is an indicator of rather constrained genetic architecture. Overall, the **G** matrices show significant differences in orientation and shape and their trajectories are influenced by selection.
2. In the third chapter, I found low male indirect genetic effects and moderate female direct genetic effects on reproductive traits of a grasshopper under a putative sexual conflict scenario over investment in current and future reproductions. Though the IGEs were initially high, they faded with time after mating.

Divergence of genetic architecture of morphology

In the first chapter of my thesis, I report the work on estimation and comparison of **G** in morphological traits of three grasshopper species, two of which, *Chorthippus biguttulus* and *Gomphocerus rufus* are rather closely related while a third species, *Pseudochorthippus parallelus*, is more distantly related (Nattier et al., 2011, Vedenina & Mugue, 2011). Such a phylogenetic trio makes it fascinating to study the structure and divergence of genetic architecture of morphological features in these grasshoppers. Morphology is largely conserved among these species from the subfamily of Gomphocerinae in the family Acrididae, except for their wings (see below). My research was driven by the question whether the **G** matrices for highly conserved traits such as morphology diverge among closely related species. I found that the **G** matrices show significant differences and have diverged from each other. The matrix comparison methods clearly point out that **G** has diverged in shape and orientation and the divergence has taken place along wing length. We performed a divergence analysis to determine whether the overall genetic covariance structure is aligned with the phenotypic divergence among the three species. We found alignment with the major axis of divergence only in the youngest species pair (*C. biguttulus* and *G. rufus*), while *P. parallelus* was oriented away from the major axis. Moreover, there was a clear phylogenetic signal in divergence of **G**, such that distantly related species have the least similar **G** matrix. Hence the alignment of **G** matrices in this study is a matter of temporal scale, for example, on a longer temporal scale, *P. parallelus* showed poor alignment with divergence. There are instances of alignment of divergence with genetic covariation in other studies, like in deeply diverged *Anolis* lizards (McGlothlin et al., 2018) or in recently radiated ecotypes of plants (Walter et al., 2018). Though there are exceptions, in crickets there seems to be no phylogenetic signal in divergence and overall rather similar structure of **G** (Bégin & Roff, 2004).

Wing is a highly variable trait in grasshoppers. The three species of grasshoppers differ markedly in wing length. *P. parallelus* has the shortest wings whereas *C. biguttulus* has the longest ones. Wing length also varies within species and between sexes, showing a high degree of sexual dimorphism. It can also be polymorphic within populations. Moreover, male grasshoppers use wings to produce sound by rubbing a raised vein of the fore wing on a stridulatory file of the femur (Uvarov, 1966). Hence wing has a major role in song production, mate recognition and attracting females. The analyses show that wing length varies independent of other traits and might be the most responsive to selection.

Besides comparing **G**, one of the objectives of this study was to estimate genetic correlations between traits. This gives a picture of the constraint in **G** and the extent of correlational selection acting on pairs of bilaterally symmetrical traits stabilizing the genetic covariance structure. Higher

correlation increases the stability of **G** and depicts similar pathway of genetic control which is true for most functionally related traits. I found overall moderate genetic correlations in all species with *P. parallelus* showing strongest correlations among all three. Hence, overall traits are not genetically constrained to a high degree and show substantial independent genetic variance. On the other hand, cross sex genetic correlations were moderate on average but were higher for lobes, eyes and femur and lower for wings and antennae. Theory predicts a negative covariation between cross-sex correlation (r_{MF}) and sexual dimorphism (Bonduriansky & Rowe, 2005). Homologous traits shared by males and females are regulated by a shared genetic machinery. This is indicated by a strong r_{MF} between traits. Due to sex-specific selection on these traits, sexes reach their specific phenotypic optima resulting in sexual dimorphism (Bonduriansky & Chenoweth, 2009). High r_{MF} of lobes, eyes and femur in the grasshoppers reflects shared genetic control of traits and hence a low degree of sexual dimorphism. Males use wings to produce sound and also use antenna in display during mating, for example in *G. rufus*. Hence due to sex specific selection these traits have different phenotypic optima in the sexes resulting in sexual dimorphism and a low $r_{MF.}$

This comparative study on the genetic architecture of morphology is an important addition to the body of empirical literature on **G** matrix stability. It is one of relatively few analyses that compare **G** matrices among multiple species. The study showed divergence of genetic variance-covariance structure for highly conserved morphological traits.

Difference in genetic architecture of male songs

In the second chapter of my thesis, I describe the work on the estimation and comparison of **G** matrices for male song traits in the grasshoppers, *Gomphocerippus rufus* and *Chorthippus biguttulus*. Features of male calling songs are important to female choice and are also used in mate recognition (Roff et al., 1999). I used five song traits, syllable duration, strophe duration, dominant frequency, onset accentuation and maximum amplitude, and estimated their additive genetic variance covariance matrix (**G**). I found significant differences between the **G** matrices in both shape and orientation. *C. biguttulus* showed tight correlations with a more constrained genetic architecture in comparison to *G. rufus.* Maximum amplitude showed strong negative correlation with strophe duration as also did onset accentuation. The phenotypic correlation for maximum amplitude and strophe duration is also negative, which implies that louder songs are of shorter duration. *C. biguttulus* songs consist of several (3-5) short strophes separated by short pauses. It is known that the later strophes are shorter and louder which conforms to the negative phenotypic correlation between the traits.

Male calling songs in insects are a group of quantitative traits under multivariate sexual selection (Higgins & Waugaman, 2004, Bentsen et al., 2006, Oh & Shaw, 2013, Ower et al., 2013). Some of male grasshopper song traits, like syllable length and maximum amplitude, are reported to be under sexual selection (Butlin et al., 1985, Charalambous et al., 1994, Klappert & Reinhold, 2003). Maximum amplitude among other traits was also the least heritable in both the species. This has practical implications for the females as it is easier to track a louder male in the wild, loudness can be under female preference. The low heritability also confirms the theoretical prediction of the erosion of genetic variance under the face of selection (Kirkpatrick & Ryan, 1991).

Hence exploring the genetic architecture of such traits is important, since response to sexual selection can be expected only in the presence of additive genetic variance of these traits. Sexual selection can promote speciation by runaway processes and inspection of **G** leads us to understand the evolutionary trajectory of male songs and how they affect phenotypic divergence. Sexual selection influence phenotypic diversification and speciation as it has the ability to lead to divergence between populations by its direct effect on mate recognition (Panhuis et al., 2001). Song traits in grasshoppers are mostly under sexual selection and they also help in mate recognition. It has also been proposed that the rapid speciation in Gomphocerine grasshoppers is a direct result of sexual selection on song traits (Vedenina & Mugue, 2011). I also find very different genetic covariance structure in the two species of grasshoppers, and such a difference in their genetic architecture might be driven by sexual selection. On the other hand, the analyses on morphological traits also showed divergence among **G** matrices. It is apparent that the **G** matrices are different and sexual and natural selection has worked in concert to cause divergence in them.

The studies depicted in the first two chapters of my thesis act in unison in the exploration of the underlying genetic basis of morphology and song traits in grasshoppers. In the first chapter I showed a phylogenetic signal in the divergence of **G** matrices for morphology and identified wing length as the most important trait in the process of this divergence. Besides being important in flight, wing is the primary trait in grasshoppers which is involved in sound production. It is therefore interesting that I have also found that the **G** matrices of song traits have diverged for two species of grasshoppers. There is a previous study which reports that maximum amplitude in *C. biguttulus* is under directional sexual selection (Klappert & Reinhold, 2003), and I also found this trait to be the least heritable. Again, some of the morphological traits are sexually dimorphic and wing length is one of them, so there is ample scope for sex-specific selection and consequent divergence. Given the diverse premises, it can be assumed that the **G** matrices of both morphology and songs are largely shaped by selection. It is more speculative to infer whether sexual or natural selection played a major role in the species divergence of this grasshopper trio. We can only conclude that natural and

sexual selection might have collectively played a role in molding the genetic architecture of morphology and songs in these Gomphocerine grasshoppers.

Male indirect genetic effects in a grasshopper

I report the study on the effect of male indirect genetic effects (IGEs) on female reproductive traits on the bow-winged grasshopper, *Chorthippus biguttulus* in the third chapter of my thesis. Given the importance of IGEs in trait expression and the possible trait coevolution between males and females, it is fascinating to understand the role of male IGEs in manipulating female fecundity traits. I found low male indirect genetic effects on the female fertility traits that hardly contribute to the phenotypic variance of these traits. The direct genetic effect of the female on these traits was moderate and was significant except in egg pod number and latency to first egg pod.

Reproduction in this grasshopper provides a promising scenario where we can expect inter-locus sexual conflict (Rowe & Day, 2006, Tregenza et al., 2006). It is reported that the males can alter female behaviour by substances transferred via spermatophore (Hartmann & Loher, 1996, 1999). Males are expected to maximize their fitness by trying to fertilize a large number of eggs and the chance of fertilization diminishes with increased time lag after copulation due to possibility of numerous mating (Gregory, 1965, Zhu & Tanaka, 2002). Female fitness, in contrast, depends upon the total number of eggs laid during her life time. This creates a situation where the male can gain fitness through manipulating the female fertility traits by increasing rate of egg production, size of eggs and decreasing the gap between copulation and oviposition. There is a potential sexual conflict where selection acts in opposing direction on the sexes (Parker, 1979). So there might be sexually antagonistic selection involved on both males and females, which eventually gives rise to sexually antagonistic coevolution. The fertility traits are expressed only in females and there are male traits which are involved in manipulating the females. Under such circumstances, I question whether the male manipulates the female through indirect genetic effects on the reproductive traits. The analyses show that the IGEs were detected right after mating, and the estimate was significant for at least egg pod length, but the effects faded out with time after copulation. Hence an evolutionary response to selection on the fecundity traits would be primarily driven by direct female additive genetic variance for those traits instead of indirectly via male IGEs.

The contribution of male IGEs to the overall phenotypic variance of the fertility traits in females is small, which means there is low heritable variation for the male manipulative traits. Males can also affect female fecundity indirectly through attractiveness. So females can allocate resources differentially based on the attractiveness of the males it has mated with (Burley, 1988). A serendipitous finding in my study was the aberrant number of eggs in egg pods of some females. In

this species, with 5-6 ovarioles per side of the ovary, a typical egg pod can contain 1-10 eggs. This number of ovarioles might be polymorphic in the population, but there were only 0.7% of females that laid 11-12 eggs per pod. Furthermore, I found seven cases where the female had laid more than 12 eggs. These females were not related and were offspring of different mothers and fathers. It is also not the case that out of these, the females which laid more than one egg pod also laid more than 12 eggs in other egg pods. Each of these females also belonged to separate cohorts, so there was no cohort effect. Such large number of eggs can be laid in an egg pod by either unusually high number of ovarioles or by producing more than one egg from a single ovariole.

Future directions

Is there any multivariate selection acting on morphological or song traits? If so, is there any evolutionary response to such selection?

The work that I describe on the genetic architecture of song and morphology in the first two chapters of my thesis has deeper implications in general. An obvious question to address as a future initiative would be to run a selection analysis on both kinds of traits in the three species of grasshoppers. Linear selection gradients, β, can be estimated from fitness data, for example survival rates, for the morphological traits. Besides β, I would also estimate the matrix of nonlinear selection gradients, γ, i.e. estimating stabilizing and correlational selection. The expectation would be high correlational selection on traits like eyes and lobes which are integrally bilaterally symmetrical traits. It would also be convenient to explore if wing length is under directional selection, given the fact that it is the most influential trait in **G** matrix divergence. An evolutionary response to selection can then be estimated. In case of little or no response, the direction of selection and g_{max} can be compared for exploring whether they act in the same direction or the angle between them is large.

I have analyzed only the male calling songs for my thesis, though I have recorded songs with females as well. Due to the presence of the female, the males might be stimulated differentially and there can be variation in song parameters. This can further be explored by estimating and contrasting **G** matrices of songs with females in the two species of grasshoppers. Again, I have estimated heritability of song traits when there were hardly a few studies which report heritability of songs in grasshoppers. Armed with this data on heritability, it would be fascinating to perform female choice experiments on grasshoppers for those traits. This will simultaneously provide the data on female choice and will also help in measuring multivariate sexual selection on these traits. Though one trait, maximum amplitude, has been shown to be under sexual selection, whether other traits involved like

strophe duration, onset accentuation or dominant frequency etc. are also subjected to selection is unknown. Moreover, there is hardly any data on nonlinear selection on grasshopper song traits. It is known that traits under sexual selection also show moderate to high additive genetic variance, which is the basis of lek paradox. This can also be tested by measuring selection on these traits, that whether song traits with high heritability are also under sexual selection. Evolutionary response to selection can then be estimated to further explore whether in presence of genetic variation and selection, there is any response at all.

An additional analysis that can be explored is the multivariate analysis of morphology and songs by estimating a **G** matrix consisting of variances and covariances between morphological and song traits. Some of the morphological traits are sexually dimorphic and are used to produce songs, like wings and femur. If these traits can be analyzed together in a multivariate animal model, one can easily explore the genetic correlations and the nature of the genetic architecture of songs and morphology, how strong the changes in song parameters are with changes in wing and femur length.

For my PhD, I have studied the comparative quantitative genetics of three species of Gomphocerine grasshoppers. It would be fascinating to look at stability of **G** at different taxonomic levels, for example among populations, among other closely related Gomphocerines and among other Acridids. Such studies will provide more information about the stability of **G** across populations, across species and across genera. It is also worth exploring whether **G** matrix divergence always carries a phylogenetic signal, though such studies would a demanding endeavor as the process of estimation of **G** matrices involve a lot of work, right from setting up breeding experiments in the lab to the measuring of the trait of interest. It would be also interesting to test for stability of **G** across time (generations) in species with short life span like *Drosophila*.

Can males influence resource allocation by manipulating females through their attractiveness?

Studying IGEs in a sexual conflict context has been quite intriguing. I have found low levels of male IGEs which were not significantly different from zero, and this was under laboratory conditions and without any information about the male. Several questions along these lines can be explored in future, I would specifically want to look at IGEs influencing resource allocation in females. IGEs can act at the level of resource allocation by females which in turn might be affected by male attractiveness. The working hypothesis would be that females mate with different males but allocate resources to the eggs differentially based on the attractiveness of the male whose sperm is used to fertilize the eggs. It has been shown that if not attractiveness as a whole but attractive traits in males

are heritable (Hedrick, 1988, Taylor et al., 2007). In grasshoppers, males produce songs to attract females and some of the song traits are under female preference. One such trait is maximum amplitude that has been shown to be under sexual selection (Klappert & Reinhold, 2003), so females prefer louder males. In the wild, males and females of *C. biguttulus* can be randomly sampled and put in large enclosures. The amplitude of male songs can be measured from the enclosures. The males and females can all be genotyped by collecting DNA (e.g. from their middle leg). This will provide the relatedness information and thus can serve as a pedigree. Eventually, the eggs laid by the females have to be collected and genotyped as well for determining paternity. Egg length, number of eggs, egg pod length and number of egg pods should be measured to estimate resource allocation. Animal models can then be fitted with proper fixed and random effects to assess whether females mated to louder males allocate more resource to eggs. One can then easily estimate the male IGEs and female direct genetic effects from the male and female pedigree. Male IGEs can act at the level of resource allocation by manipulating the females to allocate resources differentially by influence of their attractiveness, and this can be detected by measuring the fecundity traits and song attractiveness simultaneously.

Exploring the evolution of genetic architecture under a quantitative genetic framework and its implications are the main themes of this thesis. The concept of genetic architecture is complex and can be looked at from different levels. In my thesis, I have studied genetic architecture of morphology and song traits by decomposing phenotypic variance into causal components of variance. Moreover, I took a multivariate perspective, which is more complicated as traits are not independent of each other, and yet it is closer to real world. As so aptly pointed out by Marks Blows, “Adaptation is an inherently multivariate process.” (Blows, 2007). I found there is no single criterion when it comes to the stability of the additive genetic variance covariance matrix **G**, even conserved morphology can show divergence and ornamental behavioural traits (song) can have constrained genetic architecture. These empirical studies are important in uncovering the intricacies of the stability of **G**. **G** matrices evolve and eventually diverge on a long evolutionary time scale, though certain aspects of their structure might remain stable. Factors promoting stability of **G** of different quantitative characters at different time scales are largely unknown and future studies exploring those factors will deeply enhance our understanding of the evolution of genetic architecture.

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Acknowledgments

"The truth is, most of us discover where we are headed when we arrive."

-**Bill Watterson**

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"And once the storm is over, you won't remember how you made it through, how you managed to survive. You won't even be sure, whether the storm is really over. But one thing is certain. When you come out of the storm, you won't be the same person who walked in. That's what this storm's all about."
— **Haruki Murakami**

Supplementary files

Supplementary materials for manuscript I

Comparative analysis of the multivariate genetic architecture of morphological traits in three species of Gomphocerine grasshoppers

Supplementary tables

Supplementary figures

Table S1: Trait means of males and females of *Chorthippus biguttulus*, *Gomphocerippus rufus* and *Pseudochorthippus parallelus* along with SE and 95 % CI, and sexual dimorphism index (SDI) of the traits, which is one subtracted from the ratio of the mean trait value of the larger sex to the mean trait value of the smaller sex (cases where males have larger trait values are marked by asterisks).

	Trait	Male	Male V_P	Female	Female V_P	SDI
Chorthippus biguttulus	Femur	8.517 ± 0.031 (8.455-8.582)	0.220	10.091 ± 0.040 (10.011-10.168)	0.480	0.185
	Wing	12.480 ± 0.048 (12.389-12.576)	0.563	14.344 ± 0.059 (14.229-14.454)	0.988	0.149
	Antenna	7.681 ± 0.042 (7.604-7.767)	0.479	6.037 ± 0.040 (5.958-6.110)	0.423	0.272*
	Lobe	2.493 ± 0.010 (2.474-2.514)	0.025	3.087 ± 0.012 (3.065-3.109)	0.052	0.238
	Eye	1.464 ± 0.006 (1.452-1.476)	0.007	1.564 ± 0.006 (1.551-1.575)	0.007	0.068
Gomphocerippus rufus	Femur	9.503 ± 0.038 (9.429-9.574)	0.201	11.323 ± 0.062 (11.201-11.438)	0.672	0.192
	Wing	12.084 ±0.056 (11.974-12.201)	0.584	13.232 ± 0.074 (13.092-13.377)	1.181	0.095
	Antenna	9.008 ± 0.063 (8.882-9.135)	0.569	7.000 ± 0.061 (6.883-7.126)	0.630	0.287*
	Lobe	2.727 ± 0.011 (2.704-2.749)	0.022	3.410 ± 0.017 (3.376-3.443)	0.052	0.250
	Eye	1.685 ± 0.008 (1.670-1.701)	0.009	1.791 ± 0.008 (1.775-1.806)	0.009	0.063
Pseudochorthippus parallelus	Femur	8.875 ± 0.051 (8.774-8.979)	0.227	10.748 ± 0.080 (10.581-10.904)	0.529	0.211
	Wing	9.182 ± 0.096 (8.997-9.370)	0.734	7.268 ± 0.102 (7.070-7.464)	0.850	0.263*
	Antenna	8.250 ± 0.091 (8.071-8.423)	0.627	6.396 ± 0.062 (6.271-6.514)	0.405	0.290*
	Lobe	2.089 ± 0.018 (2.053-2.125)	0.030	2.644 ± 0.017 (2.608-2.677)	0.033	0.266

	Eye	1.527 ± 0.012 (1.504-1.550)	0.014	1.643 ± 0.011 (1.622-1.664)	0.014	0.076

Table S2: Correlations and test for side-differences (with 95% CI) in five bilateral morphological traits of *Chorthippus biguttulus*, *Gomphocerippus rufus* and *Pseudochorthippus parallelus.*

	Correlation				**Paired t test**			
Chorthippus biguttulus	r	t	df	p	d	t	Df	p
Male femur	0.89 (0.88, 0.91)	65.89	1084	<0.001	0.02 (-0.06, 0.02)	-0.95	2267	0.34
Male wing	0.90 (0.89, 0.91)	70.48	1104	<0.001	0.02 (-0.08, 0.04)	-0.58	2267	0.56
Male Antenna	0.80 (0.78, 0.82)	42.45	994	<0.001	-0.08 (0.02, 0.14)	2.64	2121	0.0082
Male lobe	0.54 (0.48, 0.59)	16.79	690	<0.001	-0.05 (0.04, 0.06)	6.67	1726	<0.001
Male eye	0.62 (0.58, 0.66)	22.5	798	<0.001	-0.02 (0.02, 0.03)	6.43	1899	<0.001
Female femur	0.95 (0.94, 0.96)	90.53	902	<0.001	0.04 (-0.10, 0.02)	-1.32	1956	0.19
Female wing	0.93 (0.93, 0.94)	82.34	978	<0.001	0.04 (-0.13, 0.05)	-0.89	2026	0.37
Female antenna	0.83 (0.81, 0.85)	41.71	760	<0.001	-0.05 (-0.01, 0.12)	1.77	1748	0.077
Female lobe	0.42 (0.36, 0.47)	13.07	800	<0.001	-0.08 (0.06, 0.10)	7.87	1818	<0.001
Female eye	0.72 (0.68, 0.75)	28.94	800	<0.001	-0.03 (0.02, 0.04)	6.85	1822	<0.001
Gomphocerippus rufus	r	t	df	p	d	t	df	p
Male femur	0.90 (0.89, 0.91)	56.1	734	<0.001	-0.02 (-0.03, 0.06)	0.78	1599	0.43
Male wing	0.92 (0.91, 0.93)	66.43	810	<0.001	0.02 (-0.09, 0.06)	-0.48	1675	0.63
Male Antenna	0.82 (0.80, 0.85)	40.02	760	<0.001	0.00 (-0.08, 0.07)	-0.07	1612	0.95
Male lobe	0.57 (0.51, 0.64)	14.2	410	<0.001	-0.04 (0.02, 0.06)	4.79	1107	<0.001
Male eye	0.73 (0.69, 0.77)	24.53	522	<0.001	-0.03 (0.02, 0.04)	6.3	1318	<0.001
Female femur	0.95 (0.95, 0.96)	84.11	710	<0.001	0.03 (-0.11, 0.05)	-0.75	1544	0.45
Female wing	0.95 (0.95, 0.96)	89.49	802	<0.001	0.01 (-0.11, 0.10)	-0.14	1642	0.89
Female antenna	0.89 (0.88, 0.91)	51.88	672	<0.001	-0.05 (-0.03, 0.13)	1.26	1496	0.21
Female lobe	0.61 (0.55, 0.66)	16.05	438	<0.001	-0.07 (0.04, 0.10)	5.37	1142	<0.001
Female eye	0.77 (0.73, 0.80)	27.15	510	<0.001	-0.05 (0.04, 0.06)	8.49	1238	<0.001
Pseudochorthippus parallelus	r	t	df	p	d	t	df	p
Male femur	0.92 (0.91, 0.94)	41.58	292	<0.001	0.02 (-0.10, 0.05)	-0.63	651	0.53
Male wing	0.96 (0.95, 0.97)	61.05	322	<0.001	0.03 (-0.21, 0.15)	-0.34	661	0.73
Male Antenna	0.87 (0.84, 0.90)	30.3	284	<0.001	0.01 (-0.13, 0.11)	-0.14	625	0.89
Male lobe	0.92 (0.90, 0.93)	40.25	308	<0.001	-0.01 (-0.02, 0.03)	0.6	654	0.55
Male eye	0.89 (0.87, 0.91)	36.02	328	<0.001	-0.02 (0.00, 0.04)	2.06	681	0.040
Female femur	0.95 (0.94, 0.96)	51.22	268	<0.001	-0.03 (-0.09, 0.14)	0.48	624	0.63
Female wing	0.99 (0.99, 0.99)	117.81	296	<0.001	-0.02 (-0.25, 0.30)	0.17	610	0.87
Female antenna	0.69 (0.62, 0.75)	14.98	248	<0.001	-0.03 (-0.07, 0.13)	0.56	557	0.58
Female lobe	0.85 (0.82, 0.88)	30.16	342	<0.001	0.00 (-0.02, 0.03)	0.22	695	0.82
Female eye	0.71 (0.65, 0.75)	18.68	352	<0.001	-0.02 (0.01, 0.04)	2.71	700	0.0068

Table S3: **G** matrices for *Chorthippus biguttulus*, *Gomphocerippus rufus* and *Pseudochorthippus parallelus* (3 matrices in total). Genetic variances are shown in the diagonal, covariances below the diagonal and correlations above the diagonal. Heritabilities are shown in the last row of each species.

Chorthippus. biguttulus	**Male femur**	**Male wing**	**Male antenna**	**Male lobe**	**Male eye**	**Female femur**	**Female wing**	**Female antenna**	**Female lobe**	**Female eye**
Male femur	0.078 ± 0.047 (0.019-0.222)	0.418 ± 0.298 (-0.333-0.857)	0.468 ± 0.185 (0.065-0.778)	0.726 ± 0.181 (0.258-0.946)	0.573 ± 0.196 (0.105-0.859)	0.595 ± 0.321 (-0.306-0.952)	-0.083 ± 0.407 (-0.758-0.761)	0.161 ± 0.305 (-0.446-0.714)	0.419 ± 0.309 (-0.282-0.897)	0.438 ± 0.248 (-0.107-0.863)
Male wing	0.054 ± 0.061 (-0.012-0.246)	0.137 ± 0.096 (0.022-0.427)	0.149 ± 0.266 (-0.400-0.602)	0.573 ± 0.261 (-0.096-0.924)	0.649 ± 0.200 (0.178-0.917)	0.382 ± 0.396 (-0.560-0.911)	0.474 ± 0.282 (-0.182-0.903)	-0.250 ± 0.337 (-0.779-0.514)	0.665 ± 0.214 (0.123-0.944)	0.618 ± 0.212 (0.116-0.915)
Male antenna	0.057 ± 0.037 (0.004-0.147)	0.032 ± 0.052 (-0.036-0.165)	0.175 ± 0.053 (0.086-0.297)	0.376 ± 0.220 (-0.120-0.751)	0.157 ± 0.216 (-0.290-0.553)	0.346 ± 0.285 (-0.294-0.781)	-0.024 ± 0.294 (-0.561-0.563)	0.337 ± 0.227 (-0.133-0.751)	0.172 ± 0.260 (-0.357-0.649)	0.112 ± 0.228 (-0.341-0.564)
Male lobe	0.017 ± 0.013 (0.001-0.058)	0.019 ± 0.018 (-0.001-0.074)	0.013 ± 0.011 (-0.002-0.040)	0.006 ± 0.004 (0.001-0.019)	0.735 ± 0.151 (0.379-0.919)	0.548 ± 0.366 (-0.405-0.957)	0.128 ± 0.388 (-0.617-0.845)	-0.044 ± 0.348 (-0.672-0.664)	0.663 ± 0.231 (0.071-0.952)	0.598 ± 0.215 (0.108-0.924)
Male eye	0.010 ± 0.007 (0.001-0.031)	0.014 ± 0.010 (0.002-0.043)	0.004 ± 0.006 (-0.005-0.018)	0.003 ± 0.002 (0.000-0.010)	0.003 ± 0.001 (0.001-0.007)	0.450 ± 0.382 (-0.538-0.918)	0.193 ± 0.341 (-0.474-0.814)	-0.177 ± 0.314 (-0.700-0.540)	0.681 ± 0.181 (0.250-0.933)	0.830 ± 0.099 (0.590-0.960)
Female femur	0.042 ± 0.056 (-0.002-0.230)	0.043 ± 0.074 (-0.013-0.302)	0.035 ± 0.044 (-0.007-0.167)	0.011 ± 0.016 (-0.001-0.067)	0.006 ± 0.009 (-0.002-0.037)	0.048 ± 0.072 (0.000-0.299)	0.149 ± 0.408 (-0.673-0.845)	0.156 ± 0.362 (-0.614-0.728)	0.454 ± 0.384 (-0.455-0.946)	0.405 ± 0.386 (-0.522-0.924)
Female wing	0.005 ± 0.057 (-0.064-0.187)	0.075 ± 0.078 (-0.008-0.312)	0.004 ± 0.052 (-0.074-0.143)	0.007 ± 0.017 (-0.013-0.060)	0.005 ± 0.010 (-0.007-0.034)	0.029 ± 0.066 (-0.023-0.261)	0.145 ± 0.091 (0.012-0.372)	-0.277 ± 0.304 (-0.751-0.451)	0.406 ± 0.301 (-0.248-0.905)	0.248 ± 0.316 (-0.382-0.841)
Female antenna	0.016 ± 0.030 (-0.026-0.103)	-0.015 ± 0.039 (-0.072-0.098)	0.075 ± 0.037 (0.019-0.160)	0.001 ± 0.009 (-0.012-0.026)	-0.002 ± 0.005 (-0.010-0.013)	0.016 ± 0.032 (-0.016-0.118)	-0.021 ± 0.034 (-0.074-0.077)	0.075 ± 0.037 (0.019-0.160)	-0.242 ± 0.348 (-0.772-0.554)	-0.165 ± 0.315 (-0.688-0.549)
Female lobe	0.013 ± 0.018 (-0.003-0.074)	0.023 ± 0.024 (0.001-0.107)	0.008 ± 0.014 (-0.009-0.049)	0.005 ± 0.005 (0.000-0.023)	0.003 ± 0.003 (0.000-0.013)	0.013 ± 0.023 (-0.002-0.095)	0.017 ± 0.022 (-0.004-0.092)	-0.003 ± 0.011 (-0.016-0.032)	0.008 ± 0.008 (0.001-0.035)	0.705 ± 0.158 (0.330-0.943)
Female eye	0.008 ± 0.009 (-0.001-0.037)	0.013 ± 0.012 (0.001-0.055)	0.003 ± 0.007 (-0.006-0.023)	0.003 ± 0.003 (0.000-0.011)	0.002 ± 0.001 (0.001-0.007)	0.007 ± 0.012 (-0.002-0.048)	0.007 ± 0.011 (-0.005-0.045)	-0.001 ± 0.006 (-0.009-0.016)	0.004 ± 0.004 (0.001-0.017)	0.003 ± 0.002 (0.001-0.010)
Heritability	0.335 ± 0.170 (0.087-0.790)	0.232 ± 0.141 (0.040-0.615)	0.433 ± 0.112 (0.224-0.662)	0.230 ± 0.133 (0.032-0.594)	0.355 ± 0.123 (0.158-0.665)	0.097 ± 0.132 (0.000-0.546)	0.149 ± 0.088 (0.013-0.363)	0.205 ± 0.094 (0.053-0.413)	0.279 ± 0.206 (0.053-0.937)	0.348 ± 0.181 (0.132-0.911)

Gomphocerippus rufus	Male femur	Male wing	Male antenna	Male lobe	Male eye	Female femur	Female wing	Female antenna	Female lobe	Female eye
Male femur	0.080 ± 0.035 (0.025-0.162)	-0.113 ± 0.471 (-0.829-0.761)	0.416 ± 0.195 (-0.042-0.718)	0.721 ± 0.140 (0.375-0.906)	0.529 ± 0.176 (0.107-0.792)	0.681 ± 0.190 (0.191-0.923)	-0.221 ± 0.491 (-0.866-0.818)	0.304 ± 0.259 (-0.271-0.724)	0.604 ± 0.199 (0.125-0.901)	0.486 ± 0.242 (-0.101-0.840)
Male wing	0.004 ± 0.040 (-0.041-0.126)	0.060 ± 0.068 (0.000-0.255)	0.079 ± 0.351 (-0.572-0.717)	-0.109 ± 0.470 (-0.845-0.795)	-0.036 ± 0.434 (-0.764-0.768)	-0.128 ± 0.419 (-0.778-0.718)	0.274 ± 0.409 (-0.660-0.863)	0.099 ± 0.332 (-0.534-0.714)	-0.042 ± 0.420 (-0.722-0.758)	-0.014 ± 0.439 (-0.747-0.787)
Male antenna	0.067 ± 0.046 (-0.004-0.180)	0.020 ± 0.056 (-0.048-0.183)	0.275 ± 0.111 (0.108-0.542)	0.345 ± 0.192 (-0.086-0.679)	0.373 ± 0.178 (-0.024-0.692)	0.244 ± 0.255 (-0.302-0.666)	-0.095 ± 0.373 (-0.694-0.687)	0.483 ± 0.200 (0.057-0.808)	0.186 ± 0.248 (-0.344-0.633)	0.292 ± 0.234 (-0.207-0.685)
Male lobe	0.019 ± 0.008 (0.005-0.037)	0.000 ± 0.011 (-0.016-0.030)	0.018 ± 0.011 (-0.003-0.041)	0.009 ± 0.003 (0.004-0.015)	0.791 ± 0.080 (0.604-0.913)	0.410 ± 0.273 (-0.237-0.823)	-0.328 ± 0.489 (-0.901-0.788)	0.128 ± 0.278 (-0.450-0.616)	0.631 ± 0.186 (0.179-0.898)	0.694 ± 0.169 (0.253-0.919)
Male eye	0.011 ± 0.005 (0.001-0.022)	0.001 ± 0.008 (-0.010-0.023)	0.014 ± 0.008 (-0.001-0.030)	0.005 ± 0.002 (0.002-0.009)	0.005 ± 0.001 (0.003-0.008)	0.238 ± 0.284 (-0.390-0.692)	-0.302 ± 0.451 (-0.865-0.720)	0.074 ± 0.271 (-0.467-0.543)	0.436 ± 0.219 (-0.063-0.774)	0.727 ± 0.149 (0.355-0.918)
Female femur	0.079 ± 0.051 (0.010-0.225)	0.003 ± 0.059 (-0.062-0.192)	0.060 ± 0.071 (-0.042-0.243)	0.017 ± 0.014 (-0.006-0.050)	0.008 ± 0.009 (-0.009-0.030)	0.159 ± 0.093 (0.041-0.449)	0.002 ± 0.438 (-0.768-0.816)	0.280 ± 0.252 (-0.278-0.708)	0.505 ± 0.218 (0.028-0.854)	0.291 ± 0.301 (-0.369-0.769)
Female wing	-0.006 ± 0.047 (-0.070-0.142)	0.029 ± 0.060 (-0.014-0.206)	-0.003 ± 0.064 (-0.101-0.192)	-0.006 ± 0.014 (-0.032-0.029)	-0.005 ± 0.009 (-0.023-0.018)	0.019 ± 0.075 (-0.051-0.288)	0.076 ± 0.100 (0.000-0.375)	0.017 ± 0.347 (-0.592-0.691)	-0.155 ± 0.447 (-0.796-0.793)	-0.230 ± 0.466 (-0.853-0.776)
Female antenna	0.042 ± 0.042 (-0.017-0.149)	0.018 ± 0.048 (-0.040-0.168)	0.180 ± 0.090 (0.048-0.395)	0.006 ± 0.012 (-0.014-0.034)	0.003 ± 0.009 (-0.013-0.021)	0.058 ± 0.065 (-0.023-0.243)	0.012 ± 0.055 (-0.054-0.193)	0.180 ± 0.090 (0.048-0.395)	0.130 ± 0.274 (-0.411-0.638)	0.103 ± 0.293 (-0.484-0.639)
Female lobe	0.022 ± 0.015 (0.002-0.063)	0.003 ± 0.018 (-0.017-0.061)	0.015 ± 0.020 (-0.014-0.070)	0.007 ± 0.004 (0.001-0.018)	0.004 ± 0.003 (0.000-0.011)	0.027 ± 0.024 (0.001-0.106)	0.000 ± 0.021 (-0.025-0.075)	0.010 ± 0.019 (-0.014-0.065)	0.015 ± 0.008 (0.005-0.039)	0.572 ± 0.200 (0.108-0.867)
Female eye	0.008 ± 0.007 (-0.001-0.028)	0.002 ± 0.008 (-0.008-0.028)	0.009 ± 0.009 (-0.004-0.034)	0.004 ± 0.002 (0.001-0.009)	0.003 ± 0.001 (0.001-0.006)	0.009 ± 0.012 (-0.004-0.045)	-0.001 ± 0.010 (-0.014-0.032)	0.004 ± 0.009 (-0.007-0.029)	0.004 ± 0.004 (0.000-0.016)	0.003 ± 0.002 (0.001-0.009)
Heritability	0.396 ± 0.147 (0.138-0.714)	0.103 ± 0.111 (0.000-0.427)	0.511 ± 0.165 (0.224-0.869)	0.678 ± 0.149 (0.364-0.947)	0.705 ± 0.140 (0.408-0.954)	0.245 ± 0.128 (0.069-0.621)	0.066 ± 0.084 (0.000-0.315)	0.316 ± 0.138 (0.091-0.625)	0.423 ± 0.178 (0.159-0.919)	0.365 ± 0.182 (0.101-0.857)
Pseudochorthippus parallelus	**Male femur**	**Male wing**	**Male antenna**	**Male lobe**	**Male eye**	**Female femur**	**Female wing**	**Female antenna**	**Female lobe**	**Female eye**

Male femur	0.080 ± 0.035 (0.025-0.162)	-0.113 ± 0.471 (-0.829-0.761)	0.416 ± 0.195 (-0.042-0.718)	0.721 ± 0.140 (0.375-0.906)	0.529 ± 0.176 (0.107-0.792)	0.681 ± 0.190 (0.191-0.923)	-0.221 ± 0.491 (-0.866-0.818)	0.304 ± 0.259 (-0.271-0.724)	0.604 ± 0.199 (0.125-0.901)	0.486 ± 0.242 (-0.101-0.840)
Male wing	0.004 ± 0.040 (-0.041-0.126)	0.060 ± 0.068 (0.000-0.255)	0.079 ± 0.351 (-0.572-0.717)	-0.109 ± 0.470 (-0.845-0.795)	-0.036 ± 0.434 (-0.764-0.768)	-0.128 ± 0.419 (-0.778-0.718)	0.274 ± 0.409 (-0.660-0.863)	0.099 ± 0.332 (-0.534-0.714)	-0.042 ± 0.420 (-0.722-0.758)	-0.014 ± 0.439 (-0.747-0.787)
Male antenna	0.067 ± 0.046 (-0.004-0.180)	0.020 ± 0.056 (-0.048-0.183)	0.275 ± 0.111 (0.108-0.542)	0.345 ± 0.192 (-0.086-0.679)	0.373 ± 0.178 (-0.024-0.692)	0.244 ± 0.255 (-0.302-0.666)	-0.095 ± 0.373 (-0.694-0.687)	0.483 ± 0.200 (0.057-0.808)	0.186 ± 0.248 (-0.344-0.633)	0.292 ± 0.234 (-0.207-0.685)
Male lobe	0.019 ± 0.008 (0.005-0.037)	0.000 ± 0.011 (-0.016-0.030)	0.018 ± 0.011 (-0.003-0.041)	0.009 ± 0.003 (0.004-0.015)	0.791 ± 0.080 (0.604-0.913)	0.410 ± 0.273 (-0.237-0.823)	-0.328 ± 0.489 (-0.901-0.788)	0.128 ± 0.278 (-0.450-0.616)	0.631 ± 0.186 (0.179-0.898)	0.694 ± 0.169 (0.253-0.919)
Male eye	0.011 ± 0.005 (0.001-0.022)	0.001 ± 0.008 (-0.010-0.023)	0.014 ± 0.008 (-0.001-0.030)	0.005 ± 0.002 (0.002-0.009)	0.005 ± 0.001 (0.003-0.008)	0.238 ± 0.284 (-0.390-0.692)	-0.302 ± 0.451 (-0.865-0.720)	0.074 ± 0.271 (-0.467-0.543)	0.436 ± 0.219 (-0.063-0.774)	0.727 ± 0.149 (0.355-0.918)
Female femur	0.079 ± 0.051 (0.010-0.225)	0.003 ± 0.059 (-0.062-0.192)	0.060 ± 0.071 (-0.042-0.243)	0.017 ± 0.014 (-0.006-0.050)	0.008 ± 0.009 (-0.009-0.030)	0.159 ± 0.093 (0.041-0.449)	0.002 ± 0.438 (-0.768-0.816)	0.280 ± 0.252 (-0.278-0.708)	0.505 ± 0.218 (0.028-0.854)	0.291 ± 0.301 (-0.369-0.769)
Female wing	-0.006 ± 0.047 (-0.070-0.142)	0.029 ± 0.060 (-0.014-0.206)	-0.003 ± 0.064 (-0.101-0.192)	-0.006 ± 0.014 (-0.032-0.029)	-0.005 ± 0.009 (-0.023-0.018)	0.019 ± 0.075 (-0.051-0.288)	0.076 ± 0.100 (0.000-0.375)	0.017 ± 0.347 (-0.592-0.691)	-0.155 ± 0.447 (-0.796-0.793)	-0.230 ± 0.466 (-0.853-0.776)
Female antenna	0.042 ± 0.042 (-0.017-0.149)	0.018 ± 0.048 (-0.040-0.168)	0.180 ± 0.090 (0.048-0.395)	0.006 ± 0.012 (-0.014-0.034)	0.003 ± 0.009 (-0.013-0.021)	0.058 ± 0.065 (-0.023-0.243)	0.012 ± 0.055 (-0.054-0.193)	0.180 ± 0.090 (0.048-0.395)	0.130 ± 0.274 (-0.411-0.638)	0.103 ± 0.293 (-0.484-0.639)
Female lobe	0.022 ± 0.015 (0.002-0.063)	0.003 ± 0.018 (-0.017-0.061)	0.015 ± 0.020 (-0.014-0.070)	0.007 ± 0.004 (0.001-0.018)	0.004 ± 0.003 (0.000-0.011)	0.027 ± 0.024 (0.001-0.106)	0.000 ± 0.021 (-0.025-0.075)	0.010 ± 0.019 (-0.014-0.065)	0.015 ± 0.008 (0.005-0.039)	0.572 ± 0.200 (0.108-0.867)
Female eye	0.008 ± 0.007 (-0.001-0.028)	0.002 ± 0.008 (-0.008-0.028)	0.009 ± 0.009 (-0.004-0.034)	0.004 ± 0.002 (0.001-0.009)	0.003 ± 0.001 (0.001-0.006)	0.009 ± 0.012 (-0.004-0.045)	-0.001 ± 0.010 (-0.014-0.032)	0.004 ± 0.009 (-0.007-0.029)	0.004 ± 0.004 (0.000-0.016)	0.003 ± 0.002 (0.001-0.009)
Heritability	0.396 ± 0.147 (0.138-0.714)	0.103 ± 0.111 (0.000-0.427)	0.511 ± 0.165 (0.224-0.869)	0.678 ± 0.149 (0.364-0.947)	0.705 ± 0.140 (0.408-0.954)	0.245 ± 0.128 (0.069-0.621)	0.066 ± 0.084 (0.000-0.315)	0.316 ± 0.138 (0.091-0.625)	0.423 ± 0.178 (0.159-0.919)	0.365 ± 0.182 (0.101-0.857)

Table S4: **G** matrices for *Chorthippus biguttulus*, *Gomphocerippus rufus* and *Pseudochorthippus parallelus* when estimated separately for the two sexes (3 x 2 = 6 matrices in total). Genetic variances are shown in the diagonal, covariances below the diagonal and correlations about the diagonal. Heritabilities are shown in the last row of each species.

	Males					**Females**				
Chorthippus biguttulus	**Femur**	**Wing**	**Antenna**	**Lobe**	**Eye**	**Femur**	**Wing**	**Antenna**	**Lobe**	**Eye**
Femur	0.098 ± 0.042 (0.030-0.191)	0.586 ± 0.237 (-0.084-0.871)	0.577 ± 0.148 (0.232-0.814)	0.733 ± 0.203 (0.182-0.942)	0.569 ± 0.207 (0.000-0.842)	0.021 ± 0.025 (0.000-0.090)	0.211 ± 0.399 (-0.630-0.805)	0.005 ± 0.395 (-0.763-0.723)	0.324 ± 0.439 (-0.635-0.904)	0.272 ± 0.411 (-0.633-0.864)
Wing	0.082 ± 0.053 (-0.002-0.198)	0.172 ± 0.091 (0.021-0.375)	0.344 ± 0.233 (-0.238-0.701)	0.586 ± 0.256 (-0.077-0.904)	0.602 ± 0.215 (0.085-0.908)	0.017 ± 0.030 (-0.015-0.106)	0.147 ± 0.077 (0.028-0.325)	-0.370 ± 0.263 (-0.813-0.203)	0.432 ± 0.263 (-0.138-0.840)	0.252 ± 0.272 (-0.325-0.724)
Antenna	0.081 ± 0.035 (0.018-0.153)	0.069 ± 0.053 (-0.019-0.185)	0.200 ± 0.054 (0.111-0.318)	0.479 ± 0.218 (-0.029-0.812)	0.229 ± 0.219 (-0.239-0.587)	0.002 ± 0.015 (-0.020-0.042)	-0.031 ± 0.025 (-0.082-0.018)	0.066 ± 0.033 (0.012-0.143)	-0.334 ± 0.287 (-0.832-0.285)	-0.194 ± 0.269 (-0.677-0.378)
Lobe	0.018 ± 0.011 (0.000-0.041)	0.019 ± 0.014 (0.000-0.053)	0.016 ± 0.010 (0.000-0.038)	0.006 ± 0.003 (0.000-0.014)	0.686 ± 0.205 (0.050-0.905)	0.005 ± 0.008 (-0.003-0.026)	0.014 ± 0.012 (-0.003-0.043)	-0.006 ± 0.006 (-0.017-0.007)	0.006 ± 0.003 (0.001-0.014)	0.653 ± 0.169 (0.234-0.891)
Eye	0.010 ± 0.006 (0.000-0.022)	0.013 ± 0.008 (0.001-0.031)	0.006 ± 0.006 (-0.004-0.018)	0.003 ± 0.002 (0.000-0.007)	0.003 ± 0.001 (0.001-0.005)	0.003 ± 0.004 (-0.002-0.013)	0.005 ± 0.006 (-0.005-0.020)	-0.002 ± 0.003 (-0.008-0.005)	0.003 ± 0.002 (0.000-0.007)	0.002 ± 0.001 (0.001-0.005)
Heritability	0.408 ± 0.155 (0.124-0.712)	0.288 ± 0.140 (0.040-0.574)	0.487 ± 0.108 (0.302-0.701)	0.210 ± 0.115 (0.011-0.463)	0.319 ± 0.110 (0.124-0.542)	0.045 ± 0.054 (0.000-0.197)	0.154 ± 0.078 (0.030-0.330)	0.181 ± 0.087 (0.035-0.378)	0.238 ± 0.114 (0.051-0.503)	0.305 ± 0.110 (0.112-0.545)
Gomphocerippus rufus	**Femur**	**Wing**	**Antenna**	**Lobe**	**Eye**	**Femur**	**Wing**	**Antenna**	**Lobe**	**Eye**
Femur	0.102 ± 0.046 (0.026-0.202)	0.288 ± 0.328 (-0.501-0.734)	0.505 ± 0.200 (-0.002-0.772)	0.576 ± 0.191 (0.119-0.842)	0.431 ± 0.217 (-0.097-0.742)	0.172 ± 0.091 (0.048-0.430)	0.313 ± 0.352 (-0.478-0.836)	0.138 ± 0.306 (-0.512-0.664)	0.475 ± 0.213 (0.022-0.830)	0.237 ± 0.289 (-0.379-0.753)
Wing	0.054 ± 0.057 (-0.028-0.182)	0.190 ± 0.103 (0.030-0.431)	0.372 ± 0.254 (-0.222-0.743)	-0.056 ± 0.351 (-0.750-0.509)	-0.002 ± 0.328 (-0.671-0.522)	0.062 ± 0.078 (-0.024-0.278)	0.132 ± 0.125 (0.000-0.448)	0.046 ± 0.340 (-0.639-0.651)	0.085 ± 0.379 (-0.661-0.769)	0.001 ± 0.375 (-0.667-0.733)
Antenna	0.097 ± 0.056	0.100 ± 0.079	0.321 ± 0.119	0.292 ± 0.210	0.325 ± 0.201	0.034 ± 0.058	0.015 ± 0.050	0.137 ± 0.083	0.019 ± 0.311	-0.045 ± 0.332

	(0.000-0.208)	(-0.028-0.269)	(0.122-0.577)	(-0.218-0.616)	(-0.145-0.648)	(-0.043-0.201)	(-0.057-0.151)	(0.011-0.334)	(-0.571-0.621)	(-0.675-0.598)
Lobe	0.017 ± 0.009 (0.001-0.034)	0.001 ± 0.013 (-0.019-0.028)	0.016 ± 0.012 (-0.007-0.038)	0.008 ± 0.003 (0.003-0.014)	0.781 ± 0.098 (0.549-0.920)	0.025 ± 0.022 (0.001-0.101)	0.007 ± 0.020 (-0.018-0.070)	0.004 ± 0.016 (-0.016-0.052)	0.014 ± 0.007 (0.005-0.036)	0.527 ± 0.207 (0.032-0.859)
Eye	0.010 ± 0.006 (-0.001-0.020)	0.002 ± 0.009 (-0.013-0.020)	0.013 ± 0.008 (-0.004-0.028)	0.005 ± 0.002 (0.002-0.008)	0.004 ± 0.001 (0.002-0.007)	0.006 ± 0.010 (-0.004-0.043)	0.001 ± 0.008 (-0.010-0.029)	0.001 ± 0.007 (-0.009-0.023)	0.003 ± 0.003 (0.000-0.014)	0.002 ± 0.002 (0.000-0.008)
Heritability	0.487 ± 0.186 (0.145-0.844)	0.321 ± 0.154 (0.052-0.643)	0.578 ± 0.169 (0.252-0.902)	0.617 ± 0.160 (0.289-0.902)	0.637 ± 0.161 (0.299-0.911)	0.267 ± 0.126 (0.079-0.598)	0.115 ± 0.105 (0.000-0.369)	0.245 ± 0.136 (0.021-0.545)	0.400 ± 0.157 (0.156-0.867)	0.284 ± 0.163 (0.062-0.798)

Pseudochorthippus parallelus	Femur	Wing	Antenna	Lobe	Eye	Femur	Wing	Antenna	Lobe	Eye
Femur	0.121 ± 0.055 (0.025-0.242)	0.557 ± 0.200 (0.097-0.872)	0.330 ± 0.207 (-0.094-0.731)	0.377 ± 0.388 (-0.559-0.877)	0.229 ± 0.409 (-0.654-0.871)	0.255 ± 0.201 (0.003-0.659)	0.495 ± 0.408 (-0.524-0.938)	0.522 ± 0.350 (-0.400-0.907)	0.484 ± 0.449 (-0.572-0.944)	0.380 ± 0.388 (-0.516-0.892)
Wing	0.115 ± 0.057 (0.007-0.232)	0.382 ± 0.164 (0.110-0.737)	0.548 ± 0.187 (0.132-0.852)	0.301 ± 0.391 (-0.628-0.858)	0.179 ± 0.430 (-0.716-0.854)	0.194 ± 0.205 (-0.019-0.619)	0.310 ± 0.276 (0.001-0.930)	0.449 ± 0.387 (-0.471-0.925)	0.486 ± 0.446 (-0.554-0.961)	0.353 ± 0.419 (-0.600-0.901)
Antenna	0.070 ± 0.045 (-0.014-0.159)	0.212 ± 0.099 (0.028-0.409)	0.411 ± 0.156 (0.138-0.722)	0.193 ± 0.367 (-0.579-0.805)	0.086 ± 0.403 (-0.724-0.763)	0.123 ± 0.116 (-0.009-0.351)	0.121 ± 0.127 (-0.020-0.382)	0.135 ± 0.094 (0.002-0.326)	0.465 ± 0.403 (-0.486-0.937)	0.387 ± 0.355 (-0.430-0.875)
Lobe	0.009 ± 0.011 (-0.004-0.036)	0.012 ± 0.015 (-0.009-0.050)	0.007 ± 0.012 (-0.016-0.034)	0.003 ± 0.004 (0.000-0.013)	0.180 ± 0.419 (-0.683-0.846)	0.036 ± 0.040 (-0.004-0.119)	0.039 ± 0.044 (-0.003-0.133)	0.023 ± 0.025 (-0.003-0.074)	0.009 ± 0.010 (0.000-0.031)	0.515 ± 0.375 (-0.411-0.937)
Eye	0.003 ± 0.005 (-0.004-0.016)	0.004 ± 0.008 (-0.010-0.023)	0.002 ± 0.007 (-0.011-0.020)	0.000 ± 0.001 (-0.001-0.003)	0.001 ± 0.001 (0.000-0.004)	0.017 ± 0.018 (-0.006-0.053)	0.018 ± 0.021 (-0.008-0.062)	0.012 ± 0.012 (-0.004-0.034)	0.004 ± 0.004 (0.000-0.014)	0.005 ± 0.003 (0.000-0.011)
Heritability	0.542 ± 0.213 (0.125-0.933)	0.483 ± 0.173 (0.159-0.818)	0.647 ± 0.192 (0.250-0.968)	0.107 ± 0.117 (0.000-0.406)	0.068 ± 0.082 (0.000-0.291)	0.448 ± 0.314 (0.006-0.981)	0.302 ± 0.246 (0.001-0.773)	0.457 ± 0.284 (0.006-0.939)	0.288 ± 0.272 (0.000-0.825)	0.411 ± 0.227 (0.017-0.890)

Table S5: **G** matrix for *Pseudochorthippus parallelus* estimated after exclusion of macropterous individuals. Genetic variances are shown in the diagonal, covariances below the diagonal and correlations about the diagonal. Heritabilities are shown in the last row.

Pseudochorthippus parallelus	**Male femur**	**Male wing**	**Male antenna**	**Male lobe**	**Male eye**	**Female femur**	**Female wing**	**Female antenna**	**Female lobe**	**Female eye**
Male femur	0.118 ± 0.062 (0.016-0.250)	0.548 ± 0.234 (0.006-0.889)	0.327 ± 0.225 (-0.143-0.727)	0.365 ± 0.405 (-0.580-0.899)	0.226 ± 0.447 (-0.727-0.877)	0.571 ± 0.304 (-0.201-0.934)	0.450 ± 0.356 (-0.398-0.914)	0.448 ± 0.320 (-0.291-0.891)	0.419 ± 0.410 (-0.544-0.920)	0.349 ± 0.352 (-0.453-0.868)
Male wing	0.104 ± 0.062 (0.000-0.232)	0.322 ± 0.171 (0.048-0.699)	0.473 ± 0.215 (-0.046-0.818)	0.248 ± 0.409 (-0.615-0.848)	0.195 ± 0.423 (-0.698-0.849)	0.391 ± 0.334 (-0.412-0.886)	0.465 ± 0.318 (-0.313-0.904)	0.360 ± 0.329 (-0.356-0.876)	0.304 ± 0.404 (-0.576-0.886)	0.231 ± 0.363 (-0.531-0.833)
Male antenna	0.066 ± 0.049 (-0.021-0.167)	0.165 ± 0.099 (-0.006-0.376)	0.391 ± 0.165 (0.098-0.720)	0.111 ± 0.364 (-0.606-0.743)	0.128 ± 0.375 (-0.634-0.735)	0.300 ± 0.309 (-0.381-0.794)	0.217 ± 0.323 (-0.480-0.764)	0.358 ± 0.294 (-0.300-0.811)	0.166 ± 0.361 (-0.576-0.780)	0.134 ± 0.319 (-0.498-0.711)
Male lobe	0.009 ± 0.011 (-0.004-0.035)	0.009 ± 0.014 (-0.012-0.045)	0.004 ± 0.011 (-0.019-0.030)	0.003 ± 0.004 (0.000-0.014)	0.181 ± 0.431 (-0.696-0.867)	0.335 ± 0.437 (-0.628-0.912)	0.263 ± 0.444 (-0.639-0.902)	0.275 ± 0.423 (-0.631-0.878)	0.321 ± 0.443 (-0.639-0.920)	0.221 ± 0.413 (-0.651-0.862)
Male eye	0.003 ± 0.005 (-0.005-0.015)	0.003 ± 0.007 (-0.008-0.021)	0.003 ± 0.007 (-0.007-0.023)	0.000 ± 0.001 (-0.001-0.004)	0.001 ± 0.001 (0.000-0.004)	0.162 ± 0.470 (-0.748-0.885)	0.172 ± 0.448 (-0.736-0.876)	0.174 ± 0.440 (-0.706-0.848)	0.157 ± 0.468 (-0.757-0.891)	0.153 ± 0.418 (-0.687-0.838)
Female femur	0.096 ± 0.085 (-0.004-0.294)	0.098 ± 0.106 (-0.048-0.357)	0.079 ± 0.094 (-0.075-0.309)	0.011 ± 0.017 (-0.007-0.055)	0.003 ± 0.007 (-0.009-0.021)	0.204 ± 0.178 (0.001-0.583)	0.457 ± 0.391 (-0.489-0.928)	0.523 ± 0.317 (-0.311-0.908)	0.486 ± 0.416 (-0.490-0.942)	0.376 ± 0.388 (-0.529-0.901)
Female wing	0.087 ± 0.090 (-0.024-0.299)	0.133 ± 0.124 (-0.029-0.432)	0.066 ± 0.106 (-0.118-0.310)	0.010 ± 0.017 (-0.009-0.056)	0.003 ± 0.008 (-0.010-0.023)	0.146 ± 0.171 (-0.020-0.539)	0.262 ± 0.228 (0.001-0.810)	0.395 ± 0.372 (-0.475-0.893)	0.451 ± 0.418 (-0.543-0.943)	0.311 ± 0.400 (-0.511-0.890)
Female antenna	0.060 ± 0.054 (-0.016-0.178)	0.073 ± 0.077 (-0.050-0.249)	0.136 ± 0.084 (0.003-0.310)	0.007 ± 0.011 (-0.007-0.036)	0.002 ± 0.005 (-0.007-0.016)	0.106 ± 0.101 (-0.005-0.318)	0.094 ± 0.106 (-0.031-0.327)	0.136 ± 0.084 (0.003-0.310)	0.424 ± 0.392 (-0.466-0.915)	0.382 ± 0.331 (-0.361-0.864)
Female lobe	0.016 ± 0.018 (-0.005-0.060)	0.016 ± 0.023 (-0.015-0.072)	0.009 ± 0.020 (-0.022-0.056)	0.002 ± 0.004 (-0.001-0.012)	0.001 ± 0.002 (-0.002-0.004)	0.029 ± 0.035 (-0.002-0.107)	0.030 ± 0.037 (-0.004-0.117)	0.019 ± 0.022 (-0.003-0.066)	0.008 ± 0.009 (0.000-0.028)	0.460 ± 0.380 (-0.468-0.916)
Female eye	0.008 ± 0.009 (-0.005-0.031)	0.008 ± 0.013 (-0.017-0.036)	0.005 ± 0.012 (-0.019-0.031)	0.001 ± 0.002 (-0.001-0.006)	0.000 ± 0.001 (-0.001-0.003)	0.014 ± 0.017 (-0.004-0.053)	0.014 ± 0.018 (-0.008-0.058)	0.010 ± 0.011 (-0.003-0.035)	0.004 ± 0.004 (0.000-0.014)	0.004 ± 0.003 (0.000-0.011)
Heritability	0.503 ± 0.225	0.411 ± 0.188	0.625 ± 0.215	0.106 ± 0.118	0.063 ± 0.079	0.400 ± 0.308	0.271 ± 0.214	0.476 ± 0.262	0.254 ± 0.259	0.344 ± 0.226

	(0.073-0.912)	(0.065-0.797)	(0.173-0.977)	(0.000-0.426)	(0.000-0.286)	(0.002-0.968)	(0.001-0.721)	(0.011-0.942)	(0.000-0.810)	(0.004-0.846)

Table S6: Divergence matrix **D** separately for both sexes and for both sexes combined.

Males	Femur	Wing	Antenna	Lobe	Eye
Femur	0.249	0.042	0.331	0.081	0.057
Wing	0.042	3.243	-0.034	0.515	0.027
Antenna	0.331	-0.034	0.443	0.094	0.075
Lobe	0.081	0.515	0.094	0.104	0.021
Eye	0.057	0.027	0.075	0.021	0.013
Females	Femur	Wing	Antenna	Lobe	Eye
Femur	0.380	-0.432	0.295	0.091	0.069
Wing	-0.432	14.479	-0.001	1.224	0.012
Antenna	0.295	-0.001	0.237	0.102	0.056
Lobe	0.091	1.224	0.102	0.148	0.025
Eye	0.069	0.012	0.056	0.025	0.013

Both sexes	Male femur	Male wing	Male antenna	Male lobe	Male eye	Female femur	Female wing	Female antenna	Female lobe	Female eye
Male femur	0.249	0.042	0.331	0.081	0.057	0.302	0.019	0.243	0.107	0.058
Male wing	0.042	3.243	-0.034	0.515	0.027	-0.164	6.847	0.031	0.593	0.013
Male antenna	0.331	-0.034	0.443	0.094	0.075	0.407	-0.164	0.323	0.126	0.076
Male lobe	0.081	0.515	0.094	0.104	0.021	0.065	1.067	0.078	0.124	0.019
Male eye	0.057	0.027	0.075	0.021	0.013	0.067	0.042	0.055	0.027	0.013
Female femur	0.302	-0.164	0.407	0.065	0.067	0.380	-0.432	0.295	0.091	0.069
Female wing	0.019	6.847	-0.164	1.067	0.042	-0.432	14.479	-0.001	1.224	0.012
Female antenna	0.243	0.031	0.323	0.078	0.055	0.295	-0.001	0.237	0.102	0.056
Female lobe	0.107	0.593	0.126	0.124	0.027	0.091	1.224	0.102	0.148	0.025
Female eye	0.058	0.013	0.076	0.019	0.013	0.069	0.012	0.056	0.025	0.013

Table S7: Eigendecomposition of divergence matrix **D** separately for both sexes and for both sexes combined.

Males	d_1	d_2	d_3	d_4	d_5
Eigenvalue	**3.326**	**0.726**	0.000	0.000	0.000
Femur	0.017	**0.585**	-0.649	0.000	0.486
Wing	**0.987**	-0.035	-0.097	-0.009	-0.122
Antenna	-0.004	**0.781**	0.324	-0.165	-0.507
Lobe	0.158	0.17	0.679	-0.008	0.697
Eye	0.009	0.132	0.059	0.986	-0.080

Females	d_1	d_2	d_3	d_4	d_5
Eigenvalue	**14.596**	**0.662**	0.000	0.000	0.000
Femur	-0.030	**0.745**	0.000	0.000	0.667
Wing	**0.996**	0.000	0.003	-0.078	0.044
Antenna	-0.000	**0.598**	-0.211	-0.388	-0.669
Lobe	0.084	0.259	-0.046	0.918	-0.286
Eye	0.001	0.142	0.976	-0.040	-0.158

Both sexes	d_1	d_2	d_3	d_4	d_5	d_6	d_7	d_8	d_9	D_1
Eigenvalue	**17.916**	**1.394**	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Male femur	0.002	**0.423**	0.000	0.000	0.906	0.000	0.000	0.000	0.000	0.000
Male wing	**0.425**	0.043	0.675	0.115	-0.021	-0.020	-0.001	0.028	0.475	-0.348
Male antenna	-0.009	**0.563**	0.311	-0.050	-0.263	0.067	0.006	0.168	0.017	0.694
Male lobe	0.067	0.133	-0.030	-0.647	-0.062	-0.703	-0.204	0.077	0.007	-0.113
Male eye	0.003	0.096	-0.044	-0.279	-0.045	0.474	-0.752	-0.321	0.111	-0.059
Female femur	-0.026	**0.514**	-0.310	0.042	-0.240	0.218	0.039	0.518	0.000	-0.513
Female wing	**0.899**	-0.027	-0.355	-0.005	0.010	0.065	0.016	0.021	-0.156	0.188
Female antenna	0.001	**0.412**	-0.267	0.408	-0.192	-0.331	0.025	-0.632	0.199	-0.079
Female lobe	0.076	0.176	0.386	0.006	-0.082	0.025	0.028	-0.255	-0.818	-0.267
Female eye	0.001	0.098	-0.043	-0.565	-0.046	0.337	0.625	-0.359	0.169	-0.073

Table S8: Angles between Krzanowski's common subspaces and species-specific subspaces of **G** matrices. The analysis shows redundancy in **G** matrices by reducing **G** to the k = 5 first eigenvectors to form the **H** matrix that is then subjected to Eigen analysis. The table shows posterior mean angles ± SE (95% CI) of the first five eigenvectors of **H** and the primary axis of k-dimensional subspaces of each species.

(a) Original G matrices

Eigenvectors	Eigenvalue	*Chorthippus biguttulus*	*Gomphocerippus rufus*	*Pseudochorthippus parallelus*
$\mathbf{h}_1$	2.947	5.10 ± 3.21	5.78 ± 3.40	7.41 ± 7.02
		(1.60-12.72)	(1.60-14.48)	(1.50-25.25)
$\mathbf{h}_2$	2.690	16.94 ± 11.11	10.49 ± 7.19	19.72 ± 14.95
		(3.77-48.14)	(2.92-28.52)	(3.01-57.18)
$\mathbf{h}_3$	2.530	19.25 ± 9.68	22.30 ± 10.00	22.85 ± 11.46
		(4.31-37.87)	(6.18-42.74)	(6.42-46.42)
$\mathbf{h}_4$	2.463	18.49 ± 9.66	29.55 ± 16.97	19.46 ± 11.06
		(4.35-44.19)	(5.34-62.17)	(3.98-47.15)
$\mathbf{h}_5$	2.271	17.90 ± 11.78	40.18 ± 19.72	21.71 ± 15.502
		(4.39-55.82)	(6.82-73.99)	(4.89-60.62)

(b) Mean-standardized G matrices

Eigenvectors	Eigenvalue	*Chorthippus biguttulus*	*Gomphocerippus rufus*	*Pseudochorthippus parallelus*
$\mathbf{h}_1$	2.846	9.34 ± 4.15	10.71 ± 5.78	14.48 ± 8.74
		(3.56-19.31)	(3.27-25.83)	(4.35-38.60)
$\mathbf{h}_2$	2.550	19.36 ± 11.52	14.65 ± 9.66	26.60 ± 15.26
		(5.73-52.46)	(4.49-41.83)	(7.39-63.64)
$\mathbf{h}_3$	2.327	16.90 ± 7.05	20.25 ± 7.98	41.55 ± 10.47
		(6.02-32.03)	(8.19-39.18)	(21.13-61.79)
$\mathbf{h}_4$	1.787	38.07 ± 11.08	30.79 ± 10.30	48.14 ± 13.66
		(16.47-60.52)	(13.14-51.87)	(22.91-74.04)
$\mathbf{h}_5$	1.718	35.91 ± 15.76	52.49 ± 14.68	32.69 ± 10.02
		(11.68-71.21)	(17.98-72.62)	(17.25-57.05)

Table S9: Eigenanalysis of genetic covariance tensors **Σ** for orginal and mean-standardized **G** matrices. The first eigenvectors $\mathbf{e}_{11}/\mathbf{e}_{12}$ and $\mathbf{e}_{21}/\mathbf{e}_{22}$ of the eigentensors $\mathbf{E}_1$ and $\mathbf{E}_2$ are summarized along with their trait loadings, eigenvalues and percentage of variance explained.

(a) Original G matrices				
Eigentensor	**E_1**		**E_2**	
Eigenvalue	0.373		0.041	
Variance explained	71%		7.8%	
Eigenvector	**e_{11}**	**e_{12}**	**e_{21}**	**e_{22}**
Eigenvalue	-0.98	-0.178	0.696	-0.682
Variance explained	74%	13%	40%	39%
Trait loadings				
Male femur	-0.256	-0.172	-0.233	0.227
Male wing	-0.425	0.566	-0.865	-0.177
Male antenna	-0.276	0.659	-0.219	0.332
Male lobe	-0.036	-0.048	-0.066	0.040
Male eye	-0.004	-0.022	-0.032	0.026
Female femur	-0.510	-0.305	-0.064	0.619
Female wing	-0.563	-0.286	-0.341	0.197
Female antenna	-0.300	-0.169	0.147	0.603
Female lobe	-0.088	-0.080	-0.037	0.130
Female eye	-0.038	-0.058	-0.019	0.049
(b) Mean-standardized G matrices				
Eigentensor	**E_1**		**E_2**	
Eigenvalue	1.109		0.125	
Variance explained	68%		8%	
Eigenvector	**e_{11}**	**e_{12}**	**e_{21}**	**e_{22}**
Eigenvalue	-0.975	-0.172	-0.870	0.423
Variance explained	68%	12%	50%	24%
Trait loadings				
Male femur	-0.212	-0.073	-0.870	0.423
Male wing	-0.329	0.580	0.290	-0.067
Male antenna	-0.214	0.704	0.087	-0.499
Male lobe	-0.131	-0.091	0.277	-0.223
Male eye	-0.007	-0.026	0.372	0.080
Female femur	-0.361	-0.116	0.417	0.178
Female antenna	-0.642	-0.209	0.310	-0.106
Female wing	-0.368	-0.148	-0.005	-0.791
Female lobe	-0.268	-0.157	0.337	-0.050
Female eye	-0.185	-0.223	0.403	0.042

Table S10: Flury hierarchy model comparison for orginal and mean-standardized **G** matrices. AIC values were averaged across 2,000 MCMC samples and models are ranked by decreasing AIC value. k = number of parameters contraint in a model, CPC = common principal component model, PCPC(x) = common principal component model of x equal eigenvectors.

(a) Original G matrices

Model	LogLik	k	AIC	ΔAIC
Heterogeneity	-110	0	220	
PCPC(1)	-163.6	1	329.3	109.3 ± 73.2
PCPC(2)	-211.7	2	427.4	207.4 ± 92.4
PCPC(3)	-253.1	3	512.2	292.2 ± 105.4
PCPC(5)	-289.2	5	586.4	366.4 ± 112.5
PCPC(6)	-319.2	6	648.4	428.5 ± 117.9
PCPC(4)	-345.5	4	703.1	483.1 ± 122.5
PCPC(7)	-366.0	7	746.0	526.0 ± 127.1
PCPC(8)	-378.6	8	773.1	553.2 ± 130.0
CPC = PCPC(9)	-382.8	9	783.6	563.7 ± 131.2
Proportionality = PCPC(10)	-586.4	10	1192.8	972.8 ± 261.3
Equality = PCPC(10) + Eigenvalues	-630.4	20	1300.7	1080.7 ± 268.2

(b) Mean-standardized G matrices

Model	LogLik	k	AIC	ΔAIC
Heterogeneity	-110	0	220	
PCPC(1)	-172.6	1	347.1	127.2 ± 86.3
PCPC(2)	-219.8	2	443.6	223.6 ± 98.5
PCPC(3)	-258.8	3	523.6	303.6 ± 107.3
PCPC(5)	-289.7	5	587.4	367.4 ± 113.0
PCPC(6)	-315.4	6	640.9	420.9 ± 117.0
PCPC(4)	-335.9	4	683.7	463.8 ± 120.5
PCPC(7)	-352.4	7	718.8	498.8 ± 124.9
PCPC(8)	-361.2	8	738.5	518.5 ± 127.2
CPC = PCPC(9)	-363.5	9	745.0	525.0 ± 128.5
Proportionality = PCPC(10)	-600.9	10	1221.8	1001.8 ± 261.9
Equality = PCPC(10) + Eigenvalues	-658.4	20	1356.8	1136.8 ± 273.0

Table S11: Linear model analysis predicting the magnitude and uncertainties of genetic correlations and heritabilities. Uncertainty in genetic correlations depended on species identity (larger uncertainty in species with lower sample size), but also on the magintude of the estimated genetic correlation (see also Figure S3).

(a) Response: Posterior standard deviation of genetic correlation				
	b	SE	t	P
Intercept (*biguttulus*)	0.3667	0.0166	22.08	$< 10^{-15}$
Posterior mean	-0.2669	0.0279	-9.58	**$< 10^{-13}$**
Species = *rufus*	-0.0064	0.0181	-0.35	0.72
Species = *parallelus*	0.0680	0.0181	3.75	**0.00036**
(b) Response: Average genetic correlation by trait				
	b	SE	t	P
Intercept (*biguttulus*)	0.0506	0.1082	0.47	0.64
Heritability	0.4602	0.2116	2.18	**0.039**
SE of heritability	1.2772	0.8523	1.59	0.15
Species = *rufus*	-0.1386	0.0739	-1.88	0.072
Species = *parallelus*	-0.0294	0.0838	-0.35	0.73
(c) Response: Posterior standard deviation of heritability				
	b	SE	t	P
Intercept (*biguttulus*)	0.0877	0.0170	5.16	$< 10^{-4}$
Heritability	0.1042	0.0475	2.19	**0.038**
Average genetic correlation	0.0645	0.0431	1.50	0.15
Species = *rufus*	-0.0025	0.0177	-0.14	0.89
Species = *parallelus*	0.0406	0.0171	2.38	**0.025**
(d) Response: Posterior mean of heritability				
	b	SE	t	P
Intercept (*biguttulus*)	0.0806	0.0683	1.18	0.25
Average genetic correlation	0.5316	0.1441	3.69	**0.0010**
Species = *rufus*	0.1573	0.0664	2.37	**0.026**
Species = *parallelus*	0.0939	0.0680	1.38	0.18
(d) Response: Average posterior standard deviation of genetic correlation				
	b	SE	t	P
Intercept (*biguttulus*)	0.3571	0.0173	20.60	$< 10^{-15}$
Heritability	-0.2640	0.0368	-7.17	**$< 10^{-6}$**
SE of heritability	0.3576	0.1420	2.52	**0.019**
Average genetic correlations	-0.1745	0.0319	-5.47	**$< 10^{-4}$**
Species = *rufus*	0.0286	0.0126	2.27	**0.032**
Species = *parallelus*	0.0765	0.0134	5.70	**$< 10^{-5}$**

Figure S1: Phenotypic distribution of trait values in *Pseudochorthippus parallelus*. Females are shown in grey, males in hatched pattern. Macropterous females are shown in orange and macropterous males in red.

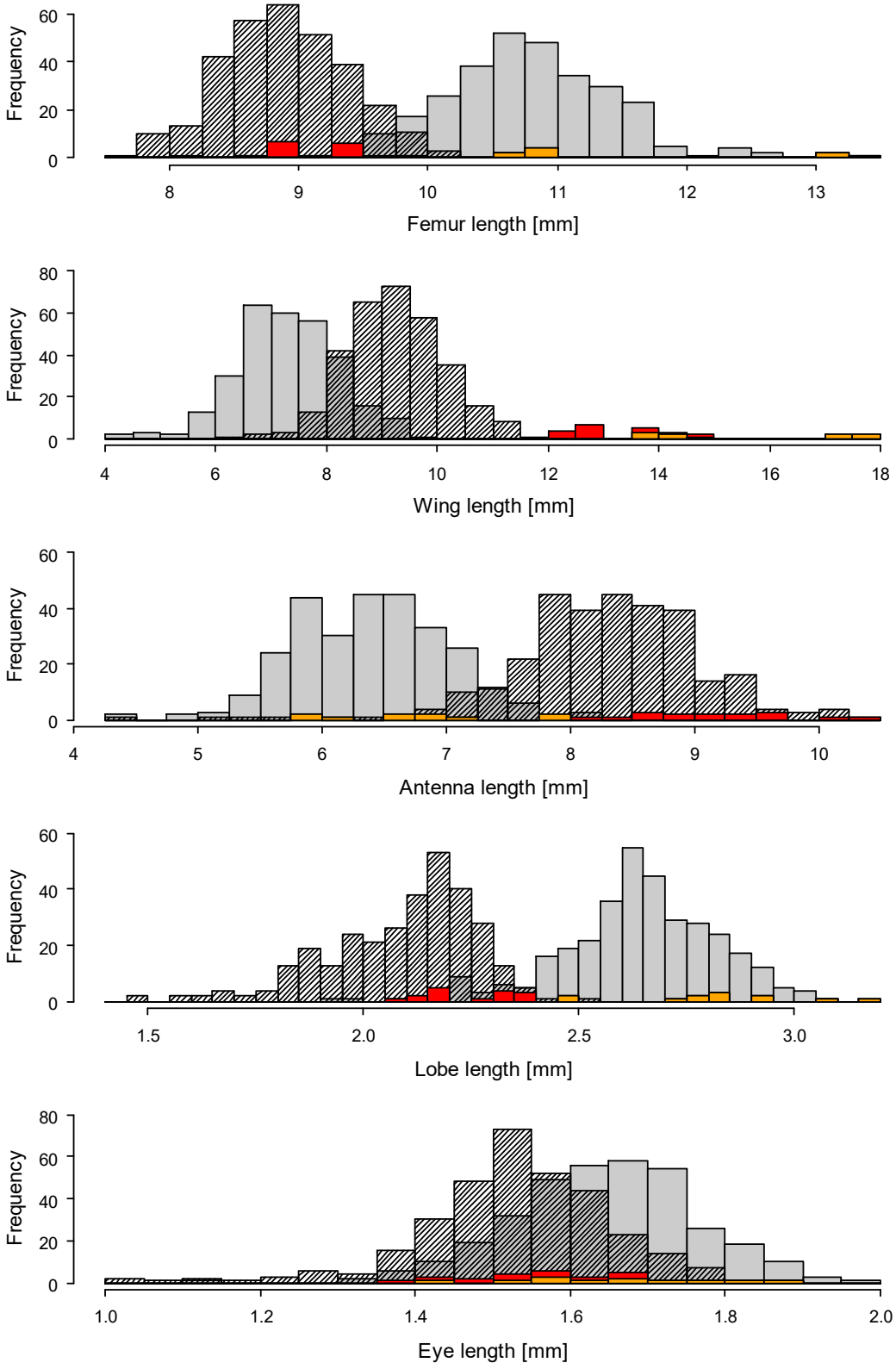


Figure S2: Phenotypic (black) and genetic (red) trait-covariation among traits in *Chorthippus biguttulus*. Each dot represents an individual. Male traits are shown above and female traits below the diagonal.

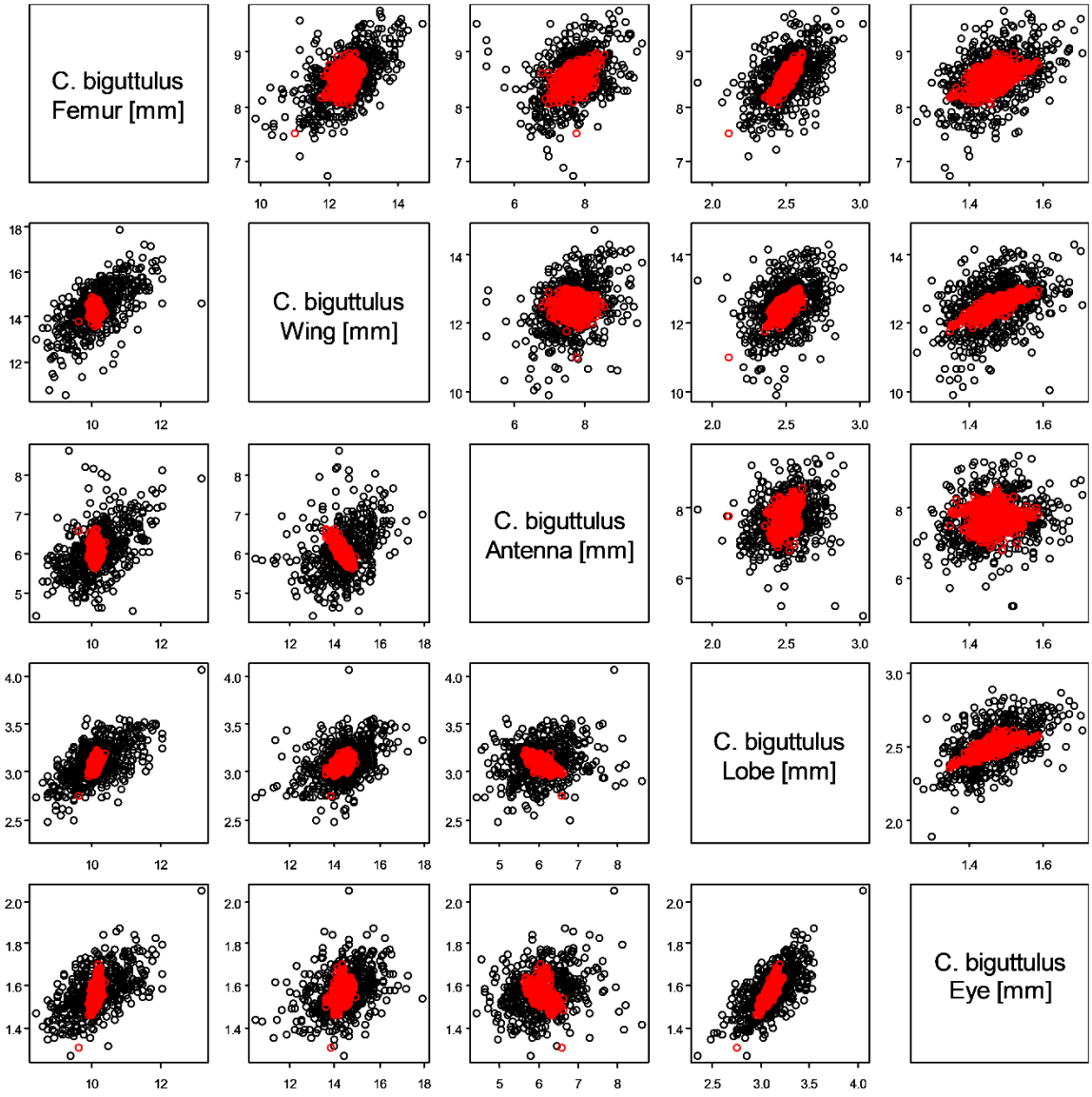


Figure S3: Phenotypic (black) and genetic (red) trait-covariation among traits in *Gomphocerippus rufus*. Each dot represents an individual. Male traits are shown above and female traits below the diagonal.

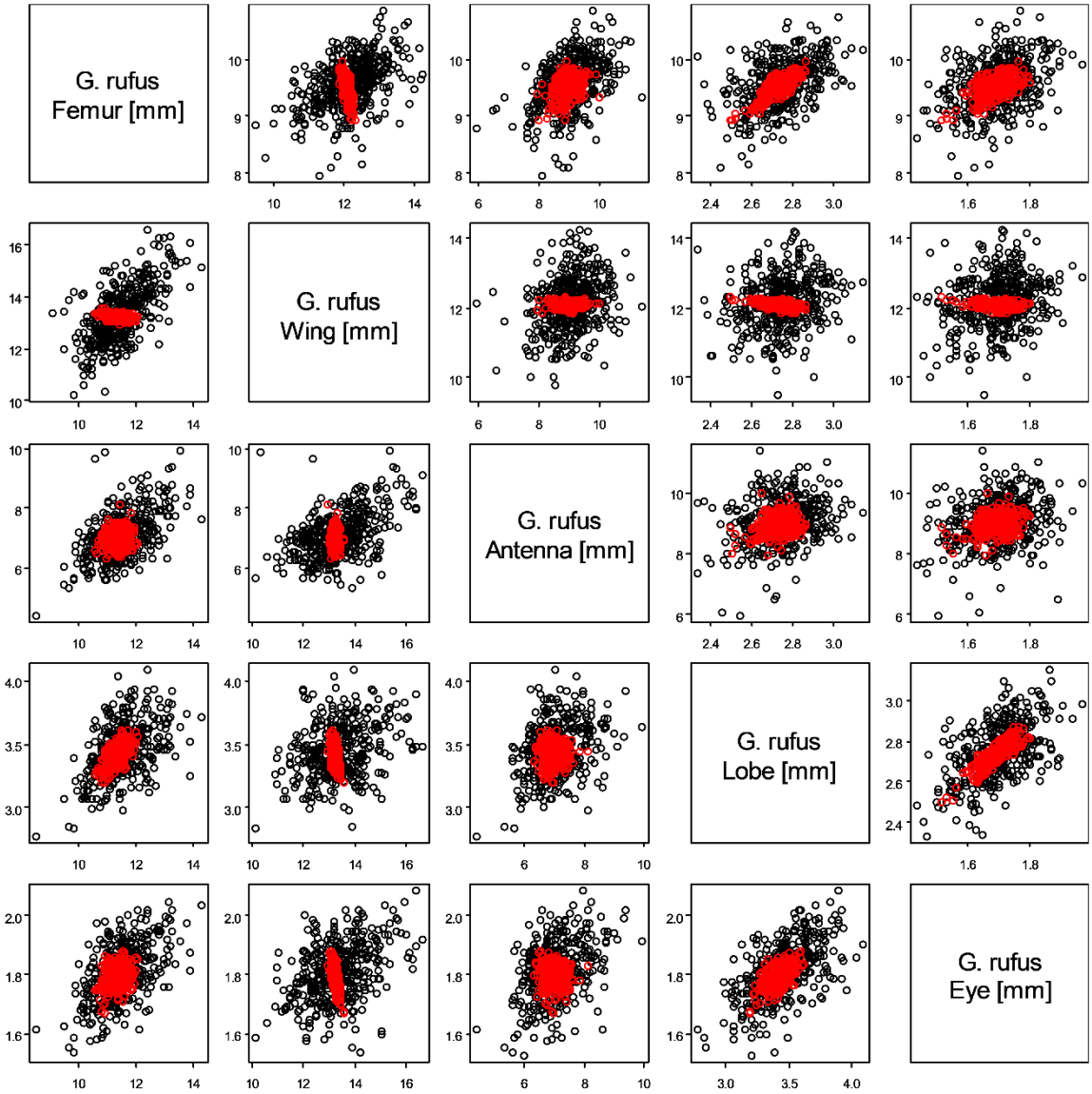


Figure S4: Phenotypic (black) and genetic (red) trait-covariation among traits in *Pseudochorthippus parallelus*. Each dot represents an individual. Male traits are shown above and female traits below the diagonal.

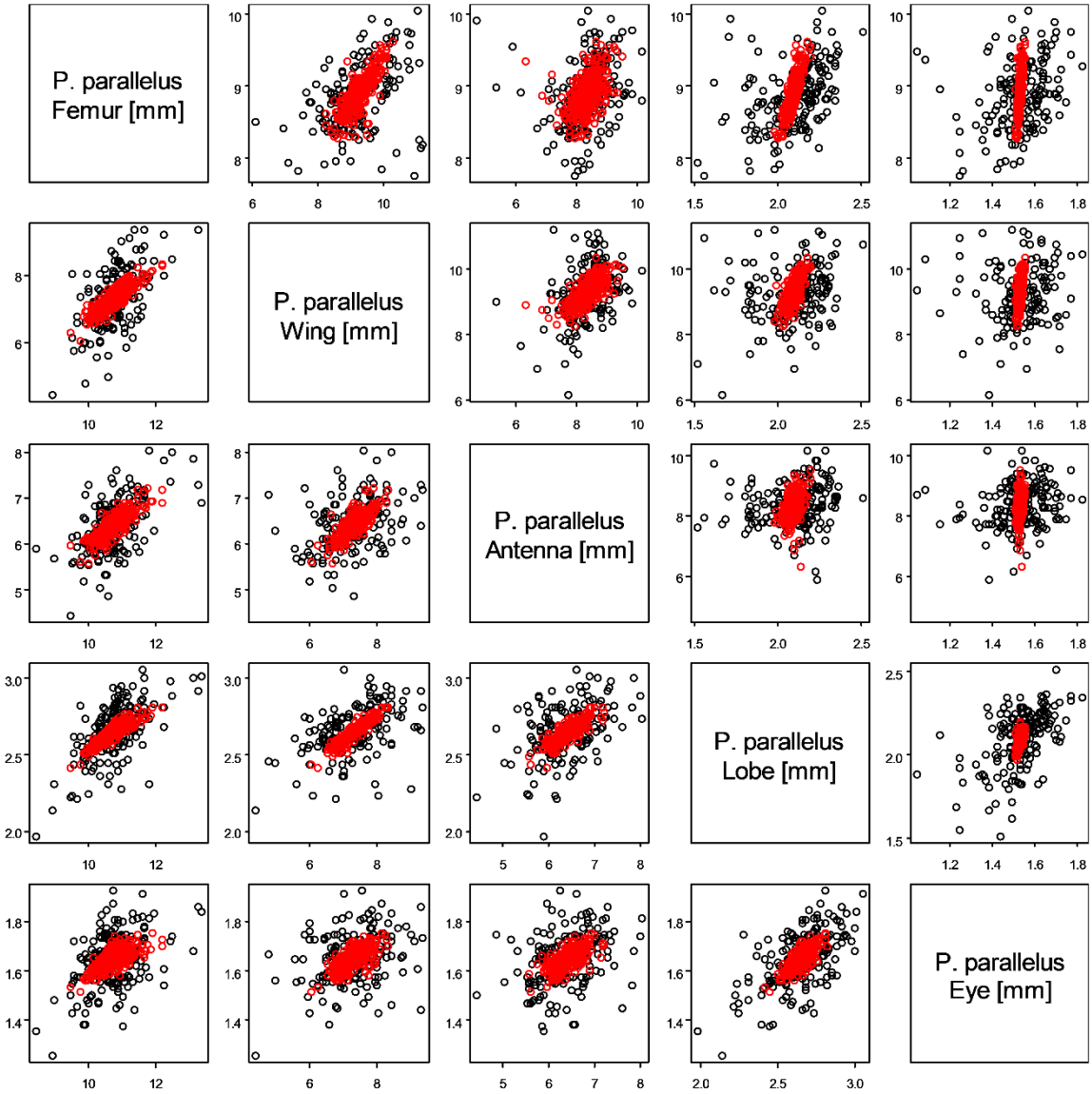


Figure S5: Prior sensitivity analysis *Chorthippus biguttulus*. Groups of bars represent from left to right (and from light to dark grey) models fitted with ν = 9, ν = 10, ν = 11 and ν = 12.

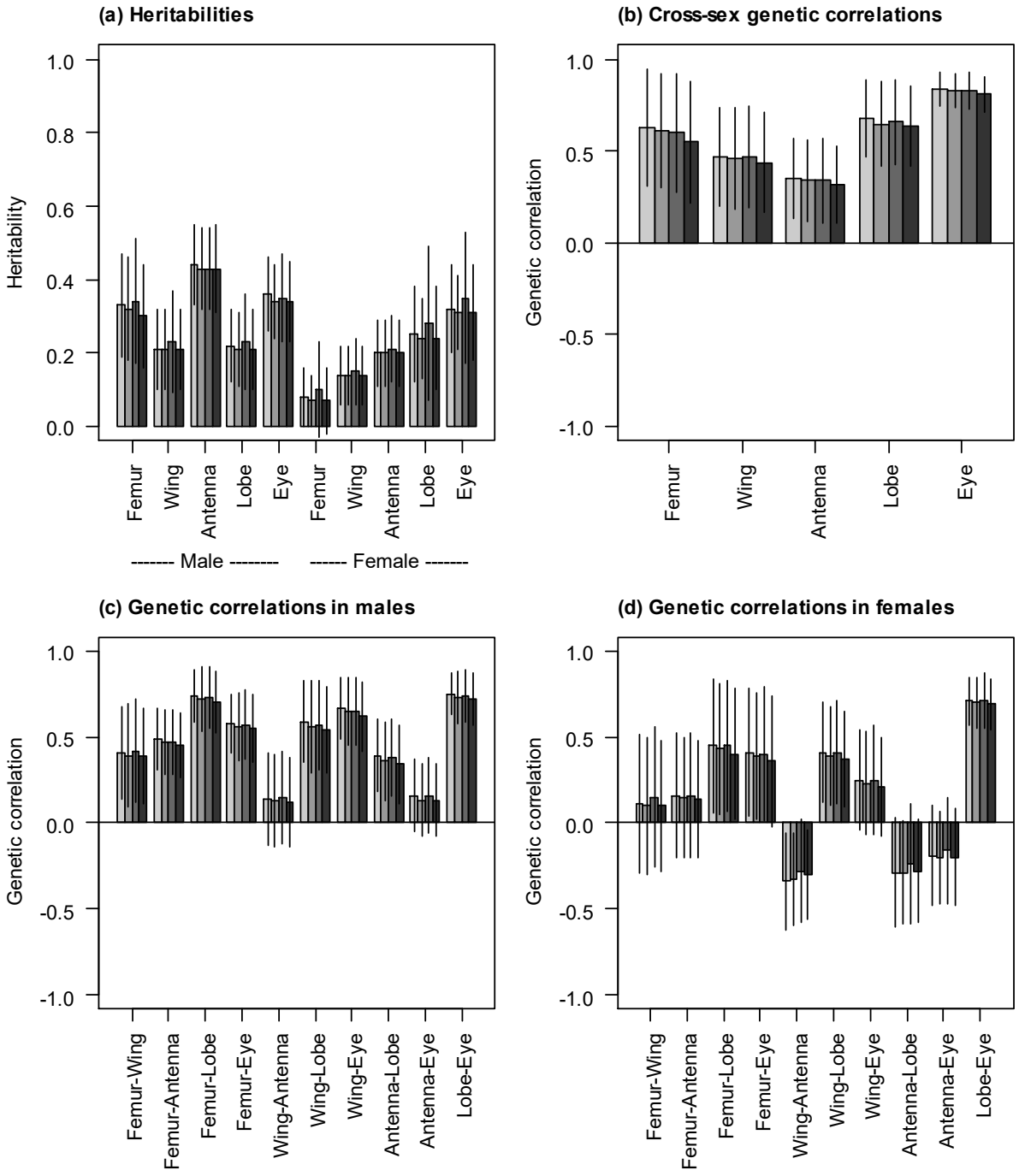


Figure S6: Prior sensitivity analysis *Gomphocerippus rufus*. Groups of bars represent from left to right (and from light to dark grey) models fitted with ν = 9, ν = 10, ν = 11 and ν = 12.

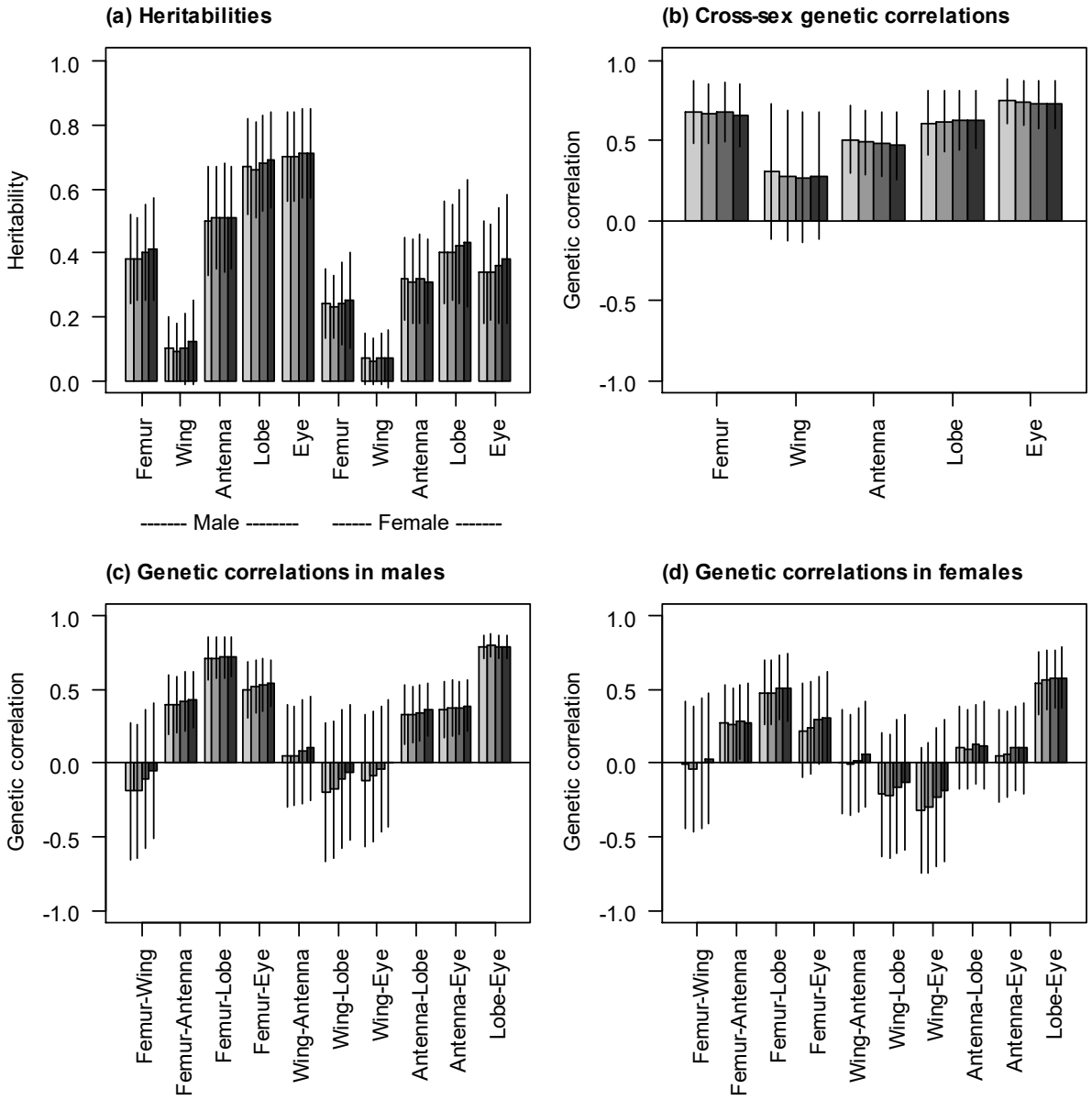


Figure S7: Prior sensitivity analysis *Pseudochorthippus parallelus*. Groups of bars represent from left to right (and from light to dark grey) models fitted with ν = 9, ν = 10, ν = 11 and ν = 12.

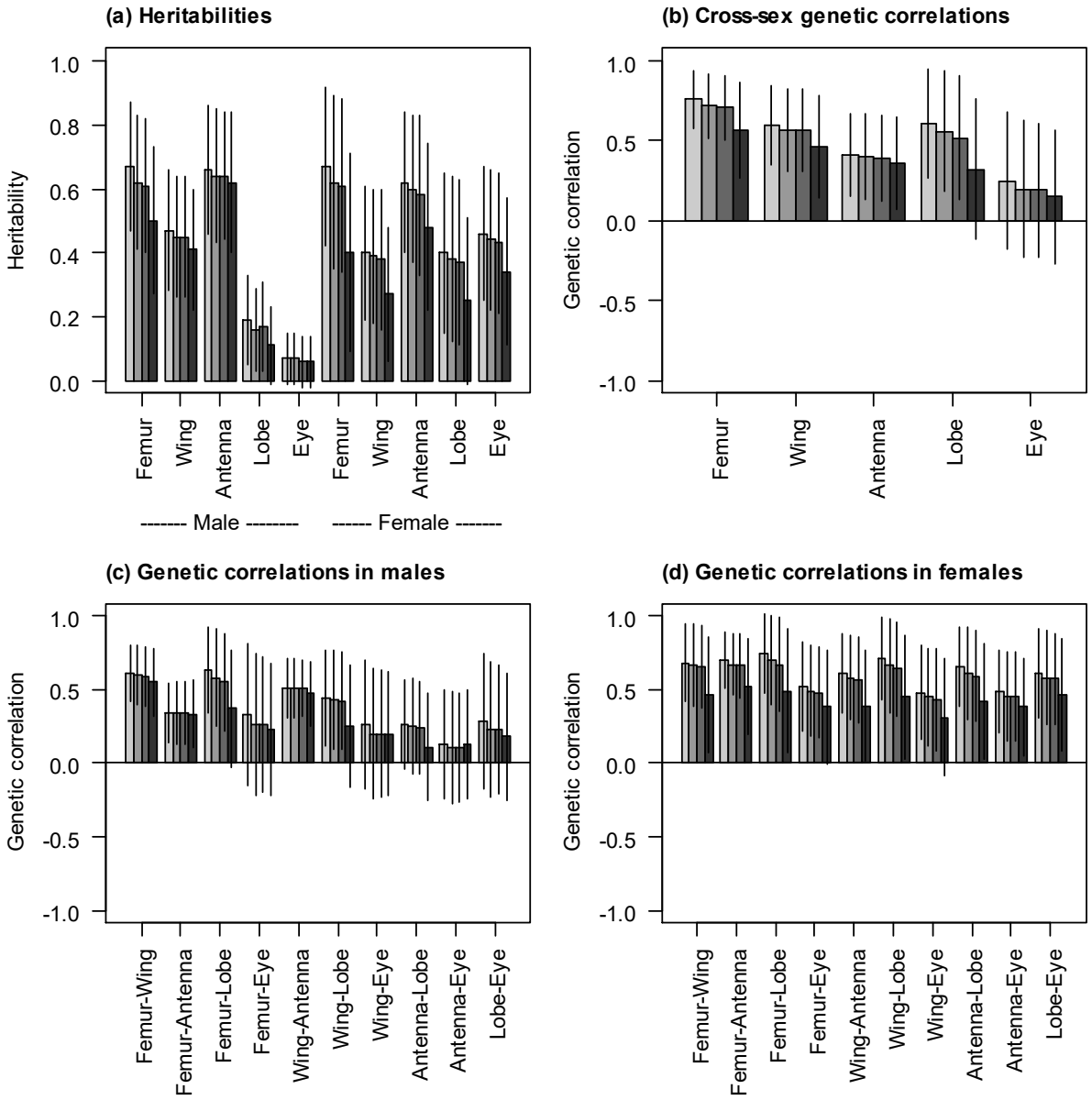


Figure S8: Covariance between posterior mean and posterior standard deviations across genetic correlations of three species of grasshoppers. Within-sex cross-trait correlations are shown as circles, cross-sex within-sex correlations as diamonds. *Chorthippus biguttulus* is shown in blue, *Pseudochorthippus parallelus* in green, and *Gomphocerippus rufus* in maroon.

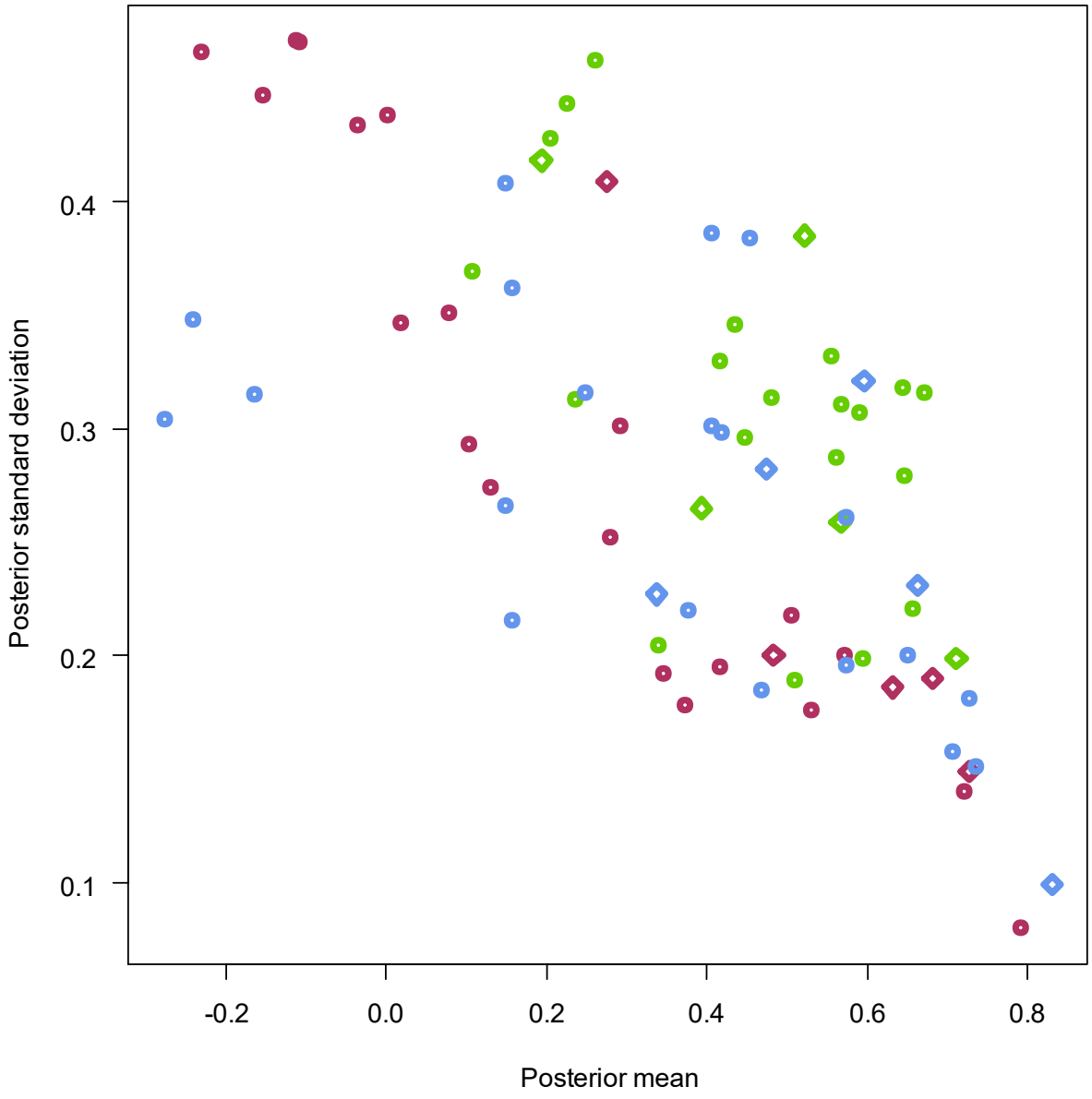


Figure S9: Covariance between estimates and their uncertainties for heritabilities and genetic correlations. *Chorthippus biguttulus* is shown in blue, *Pseudochorthippus parallelus* in green, and *Gomphocerippus rufus* in maroon.

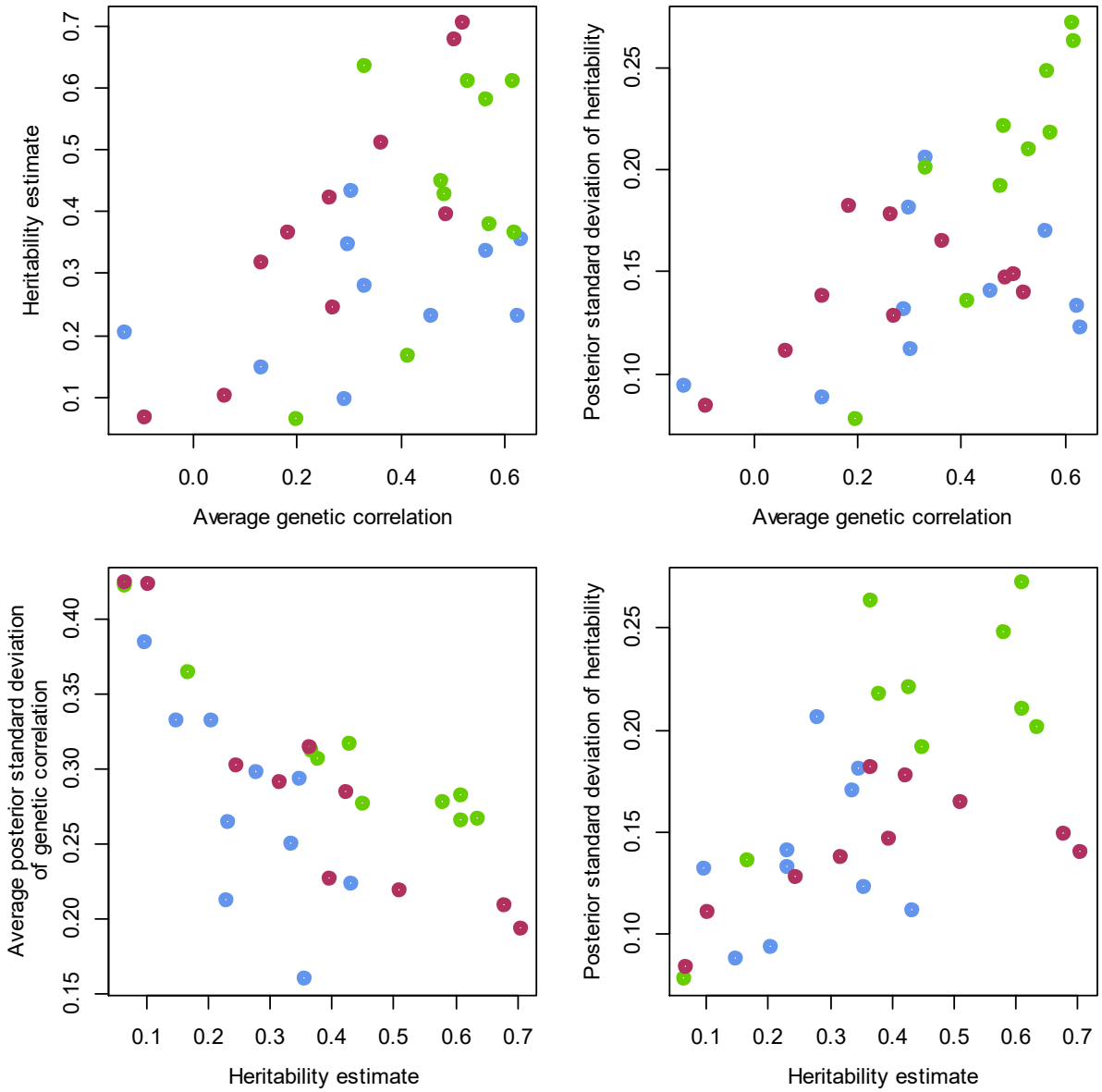


Figure S10: Contemporary genetic variation projected into divergence space among the three species of grasshoppers separated by sex. The axes show the first two principal components of the species divergence matrix **D**. Breeding values of the three species are plotted onto the same plane. Ellipses around breeding values show 95% confidence level. Lines indicated the main direction of divergence as they connect the center of the more clostly related *Chorthippus biguttulus* and *Gomphocerippus rufus* as well as the midpoint of these to species to the center of *Pseudochorthippus parallelus*.

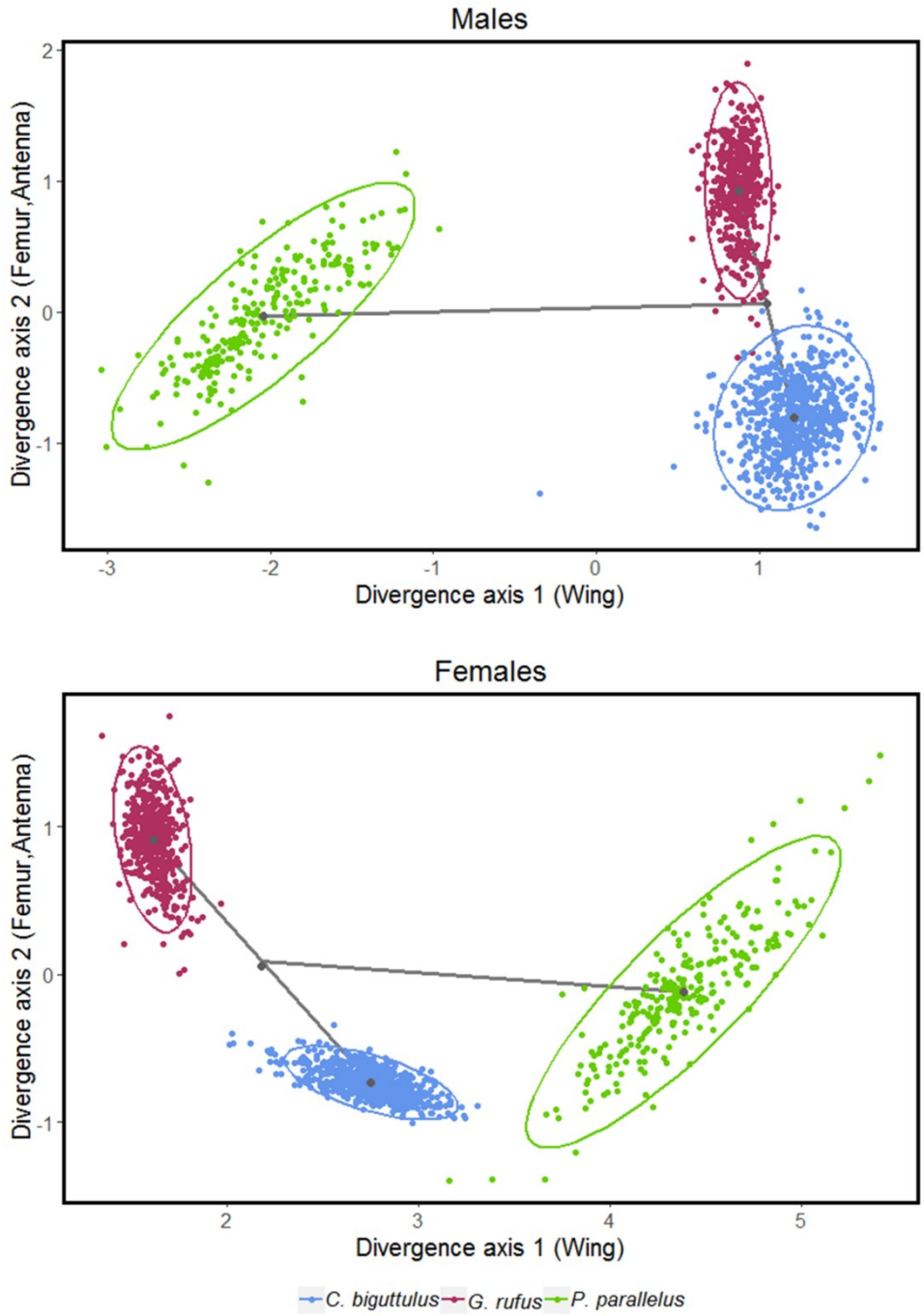


Figure S11: Pairwise trait divergences and within-species genetic covariance in females of three species of grasshoppers

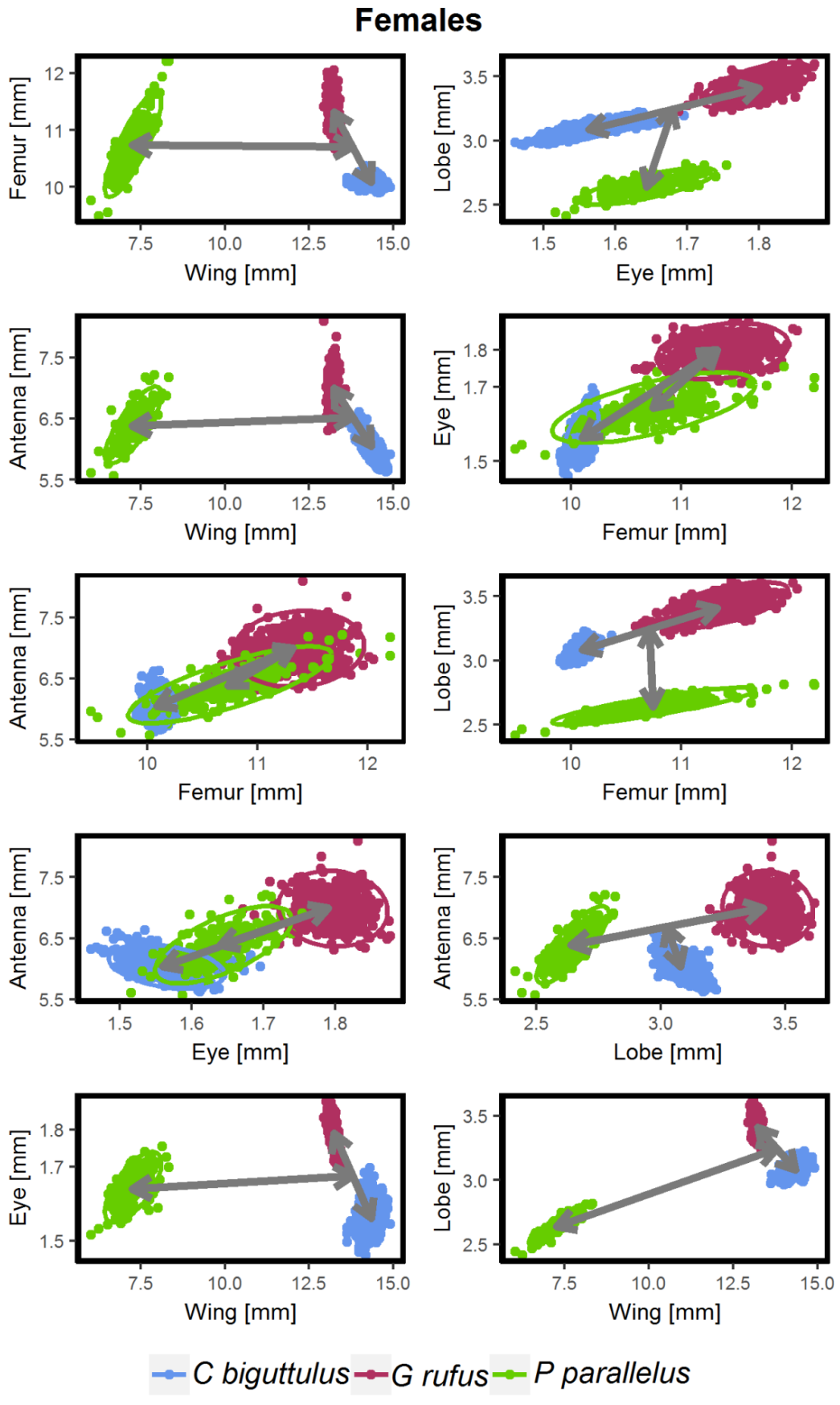


Figure S12: Pairwise trait divergences and within-species genetic covariance in males of three species of grasshoppers

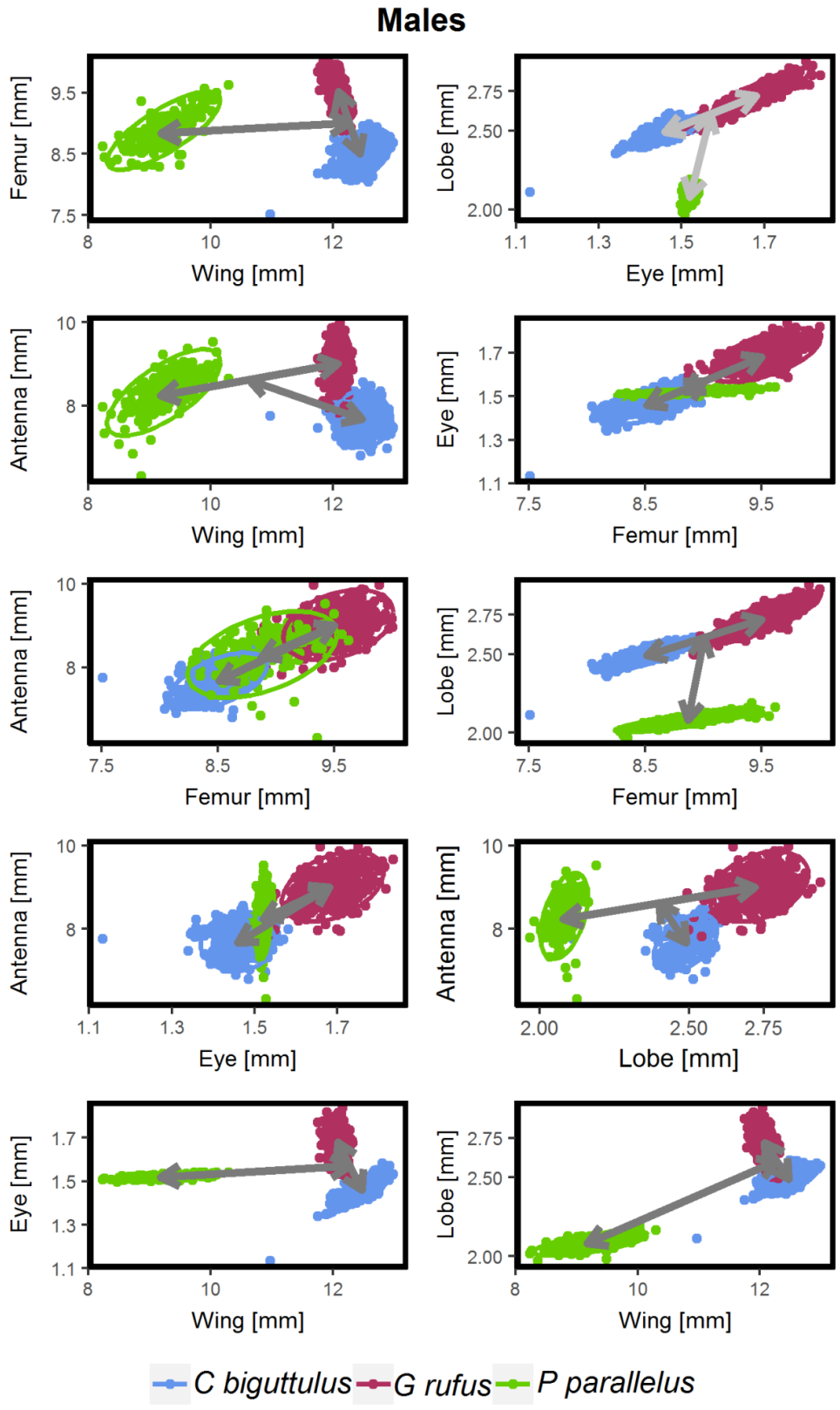
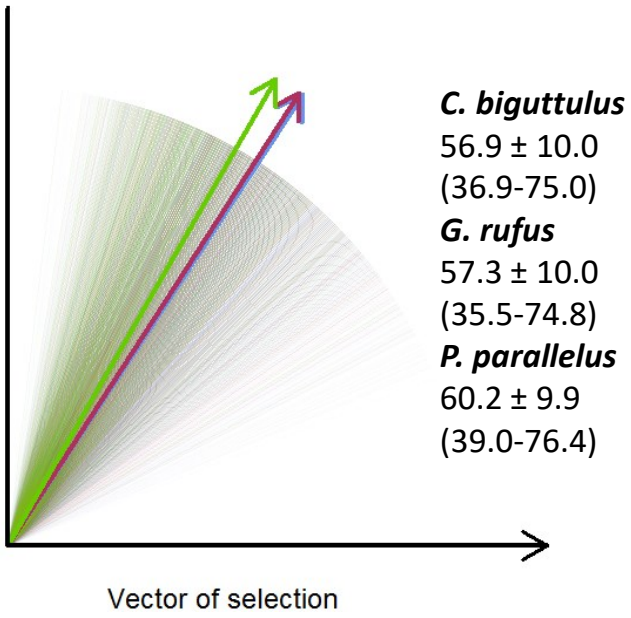


Figure S13: Random skewer projections through **G** matrices for (a) orignal **G** matrices and (b) mean-standardized **G** matrices. The figure illustrates the deflection angles between the vector of selection and the response vector based on 2,000 random skewer projections. Thin lines show individual deflection angles, while the mean angle is shown in bold for each species (blue for *Chorthippus biguttulus*, maroon for *Gomphocerippus rufus*, green for *Pseudochorthippus paralellus*). Estimates show mean ± SE (95% CI) for the angle by species.

(a) Original G matrices



(b) Mean-standardized G matrices

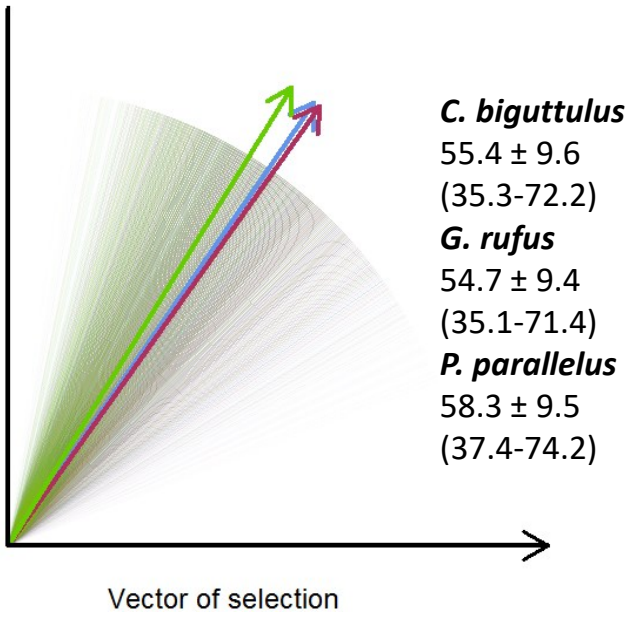
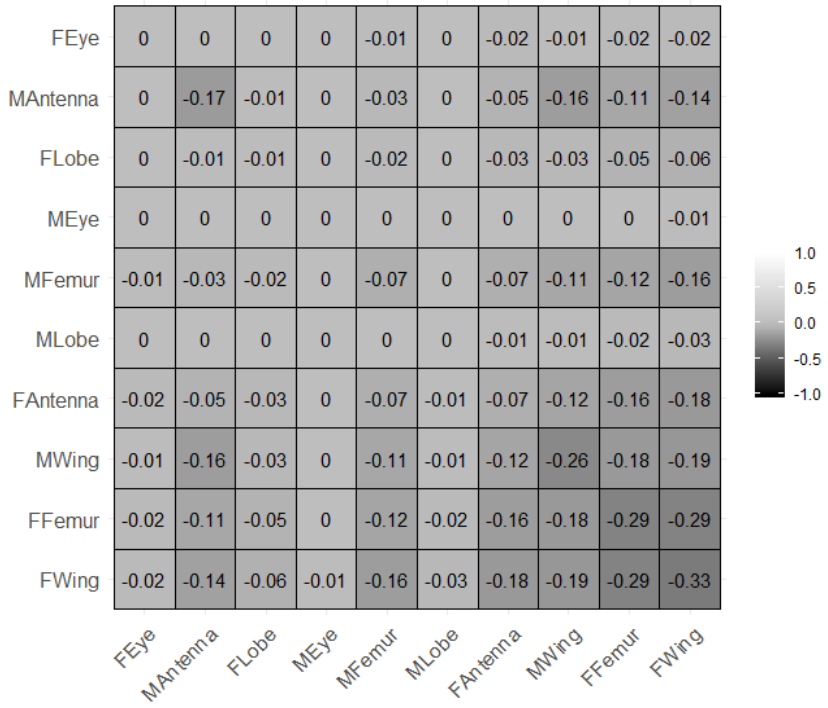


Figure S14*:* Heatmap of major genetic covariance tensor E1 of (a) orginal G matrices and (b) mean mean-standardized G matrices. Darker shades reveal greater variation across G matrices of three grasshopper species. M = male, F = female.

(a) Original G matrices



(b) Mean-standardized G matrices

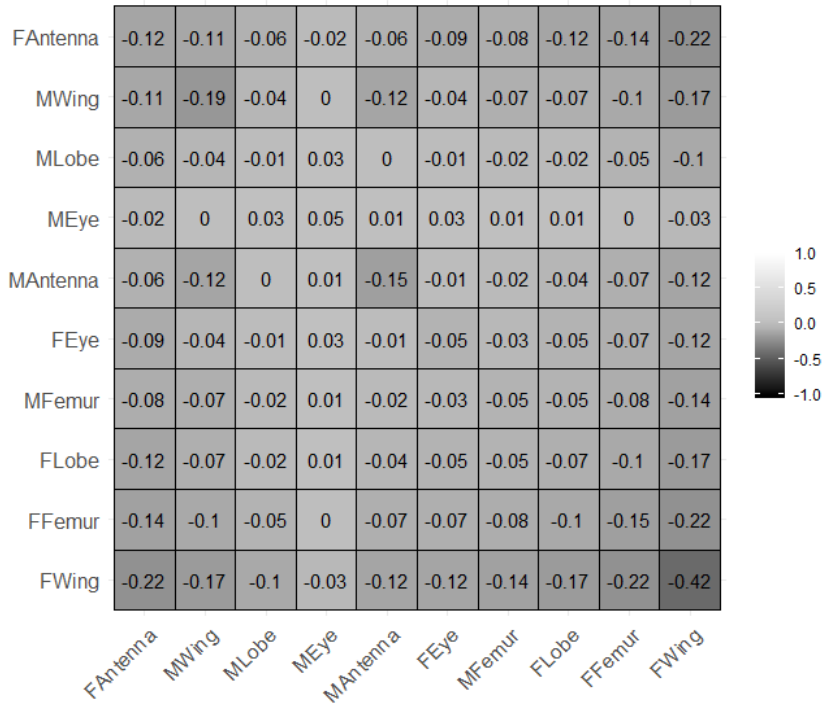


Figure S15: Genetic covariance tensor analysis based on mean-standardized G matrices. A) Variance in variation among G matrices as explained by the eigentensors of Σ as explained by the two eigentensors E_1 and E_2 of Σ. Observed values (filled symbols) were compared to values after randomization (open symbols). B) and C) Eigenanalysis of each E_1 identifies the major axis of genetic variation among G matrices. Figures show the among of additive genetic variance in each species along the major axes e_{11} of E_1 and the major axis $e_{21}$of E_2.

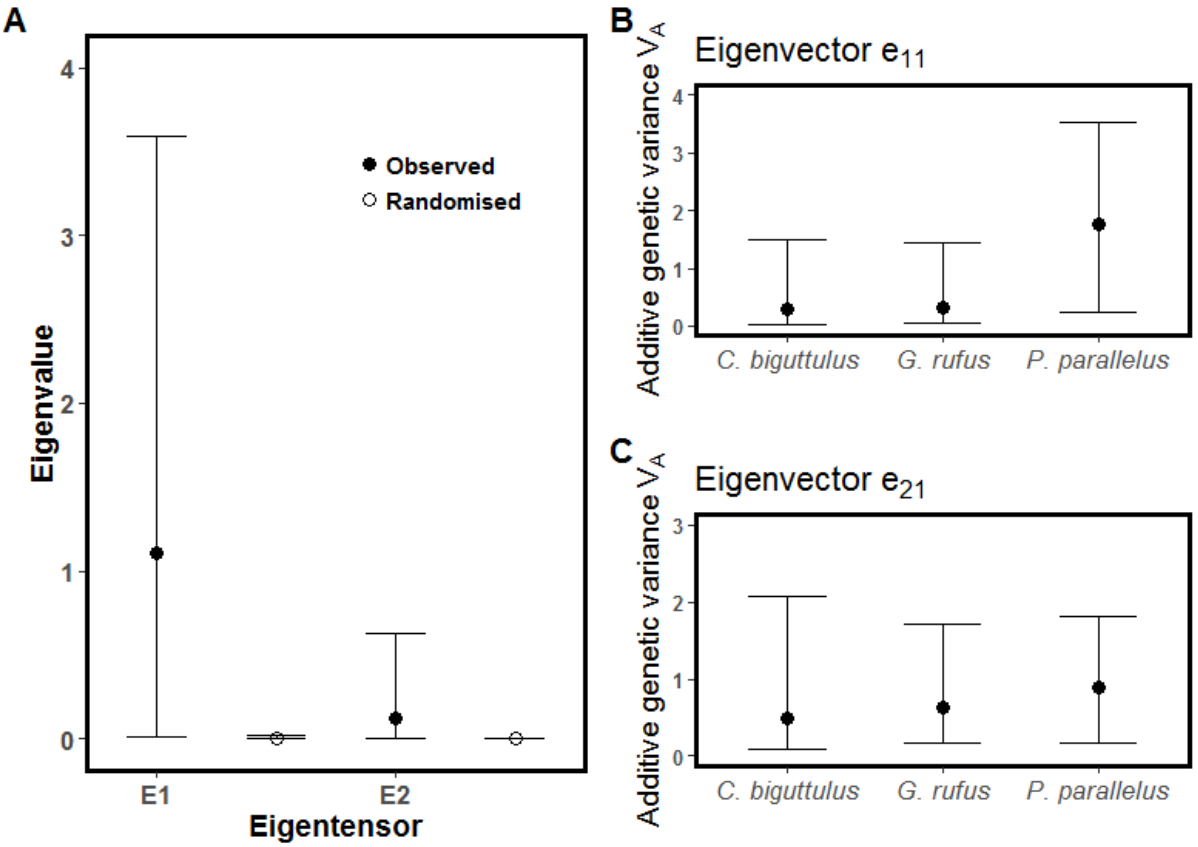
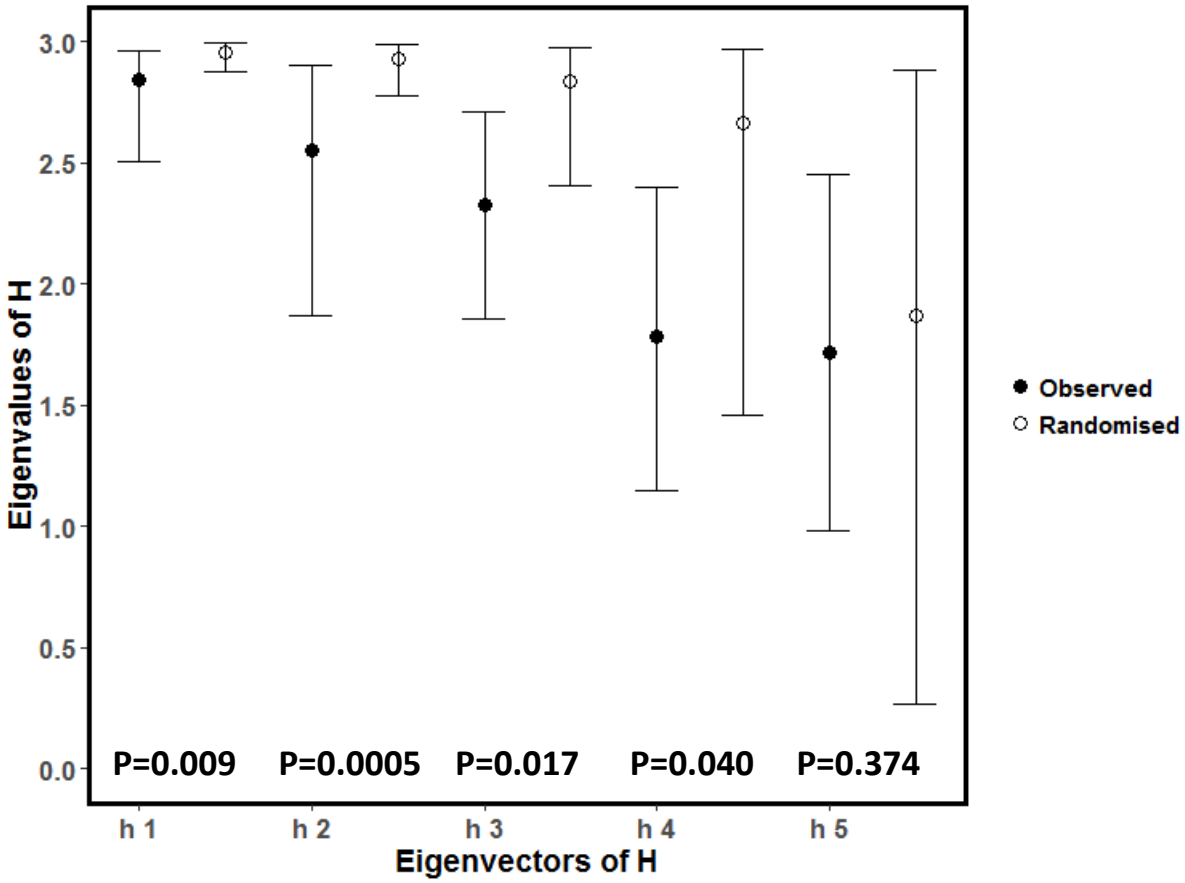


Figure S16: Krzanowski's subspace **H** for the comparison of **G** matrix among three species of grasshoppers based on mean-standardized **G** matrices. The x-axis denotes the five eigenvectors of the **H** matrix and the y-axis denotes the eigenvalues $\mathbf{h}_1$ to $\mathbf{h}_5$ of **H**. Filled symbols show empirical estimates with 95% CI and open symbols show randomized values. P values which denote the proporation of randomized values that show equal or lower values than empicial estimates (incorporating varibility in both empirical and randomized values).



Supplementary materials for manuscript III

Direct and indirect genetic effects on reproductive investment in a grasshopper

Table S1: Overview of the seven unusual cases in which females laid more than 12 eggs in a single egg pod. The table shows the laying history and some fecundity data. Within cells, data are shown in the sequence of laying.

Female	Mother	Father	Cohort	Egg pods produced	Eggs per pod	Day of laying	Average egg length (by egg pod)
M174	BM313	BHSF13	2	3	6, 13, 9	4, 7, 13	3.60, 3.30, 3.41
M186	BM054	BHSF54	2	2	15, 7	7, 12	3.67, 3.16
M179	BM147	BHSF47	2	3	14, 7, 7	5, 10, 14	3.25, 3.60, 3.62
M317	BM046	BHSF46	3	1	13	4	3.86
M438	BM218	BHSF18	3	1	19	3	3.70
M465	BM313	BHSF13	5	1	16	2	3.90
M481	BM157	BHSF57	5	2	14, 5	5, 9	3.56, 3.68

Table S2: Overview of animal models fitted for each of the five fecundity traits.

Response	Fixed effects	Random effects	Error distribution (link function)	Subsetting for temporal analysis
Egg length	Days after mating	Female pedigree + Male pedigree + Pair identity + Cohort identity + Egg pod identity	Gaussian	15 separate models for all data collected up to (1) day 1 to (15) day 15 after mating
Egg pod length	Days after mating	Female pedigree + Male pedigree + Pair identity + Cohort identity	Gaussian	
Number of eggs per egg pod	Days after mating	Female pedigree + Male pedigree + Pair identity + Cohort identity	Gaussian	
Number of egg pods	Died (yes/no) + Day of death	Female pedigree + Male pedigree + Cohort identity	Poisson (log)	15 Separate models counting all egg pods laid up to (1) day 1 to (15) day 15 after mating
Latency to first egg pod	-	Female pedigree + Male pedigree + Cohort identity	Poisson (log)	Full dataset only

Figure S1: Posterior distributions of the male pedigree effect on egg length for all subsets of the data (see main text for details on models). The light blue line shows the pooled posterior distribution of 100 independent randomizations of male pedigree links and the dark blue line shows the shows the distribution of posterior means across the 100 independent randomizations. The red vertical line shows the posterior mean for the original data. Randomization were performed for day 1, 3, 5, 10,15.

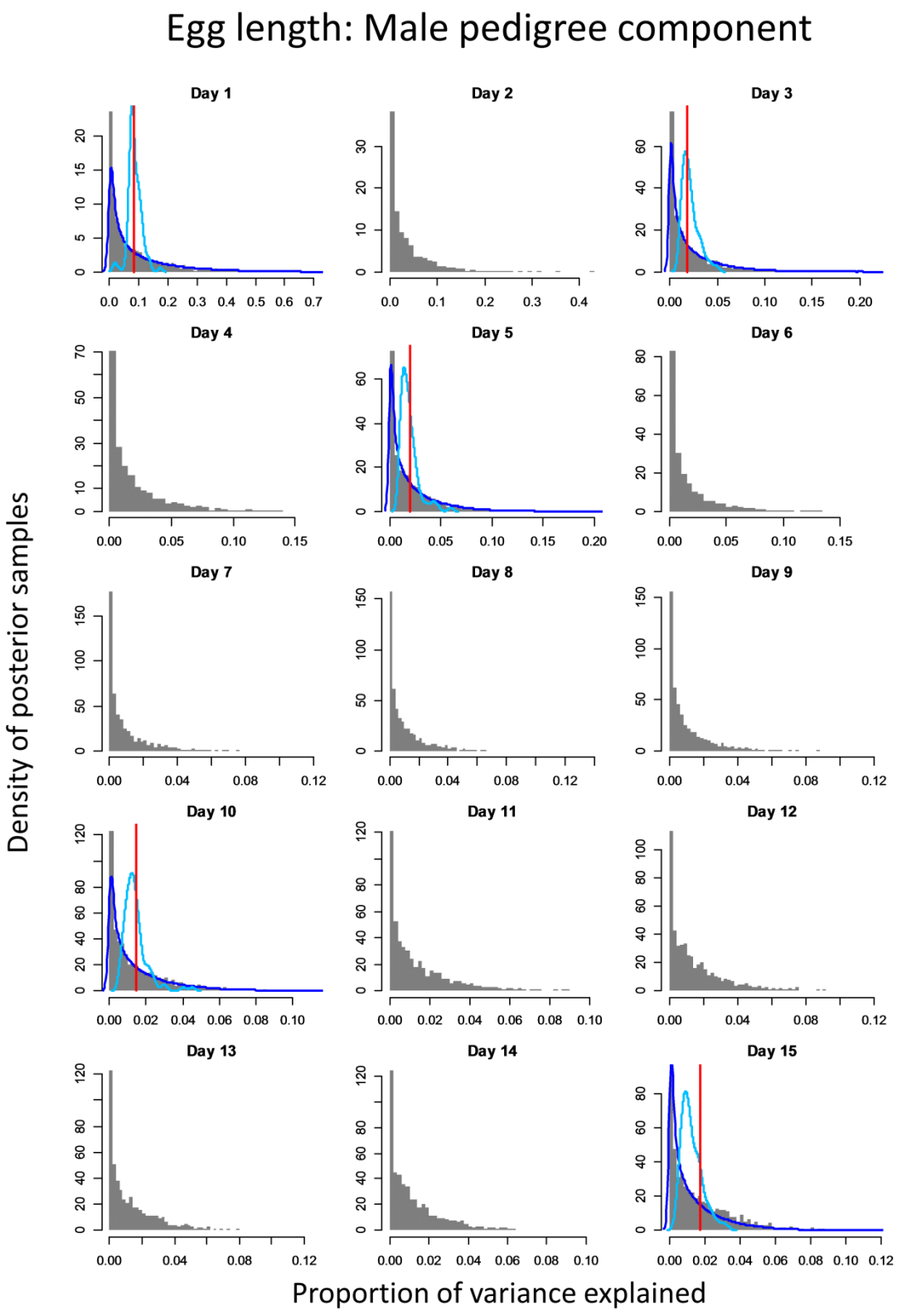


Figure S2: Posterior distributions of the male pedigree effect on egg pod length for all subsets of the data (see main text for details on models). The light blue line shows the pooled posterior distribution of 100 independent randomizations of male pedigree links and the dark blue line shows the shows the distribution of posterior means across the 100 independent randomizations. The red vertical line shows the posterior mean for the original data. Randomization were performed for day 1, 3, 5, 10,15.

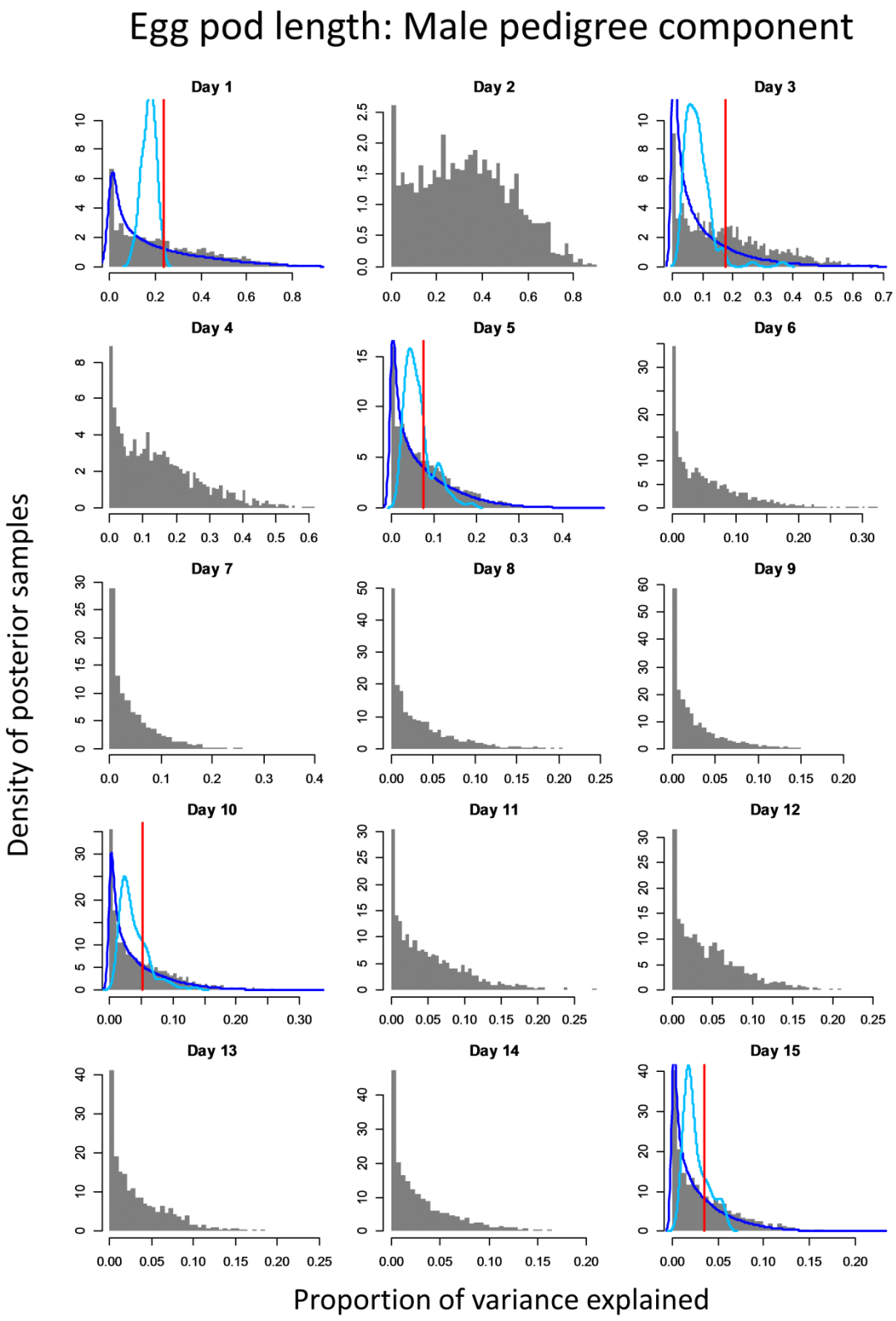


Figure S3: Posterior distributions of the male pedigree effect on eggs per egg pod for all subsets of the data (see main text for details on models). The light line shows the pooled posterior distribution of 100 independent randomizations of male pedigree links and the dark blue line shows the shows the distribution of posterior means across the 100 independent randomizations. The red vertical line shows the posterior mean for the original data. Randomization were performed for day 1, 3, 5, 10,15.

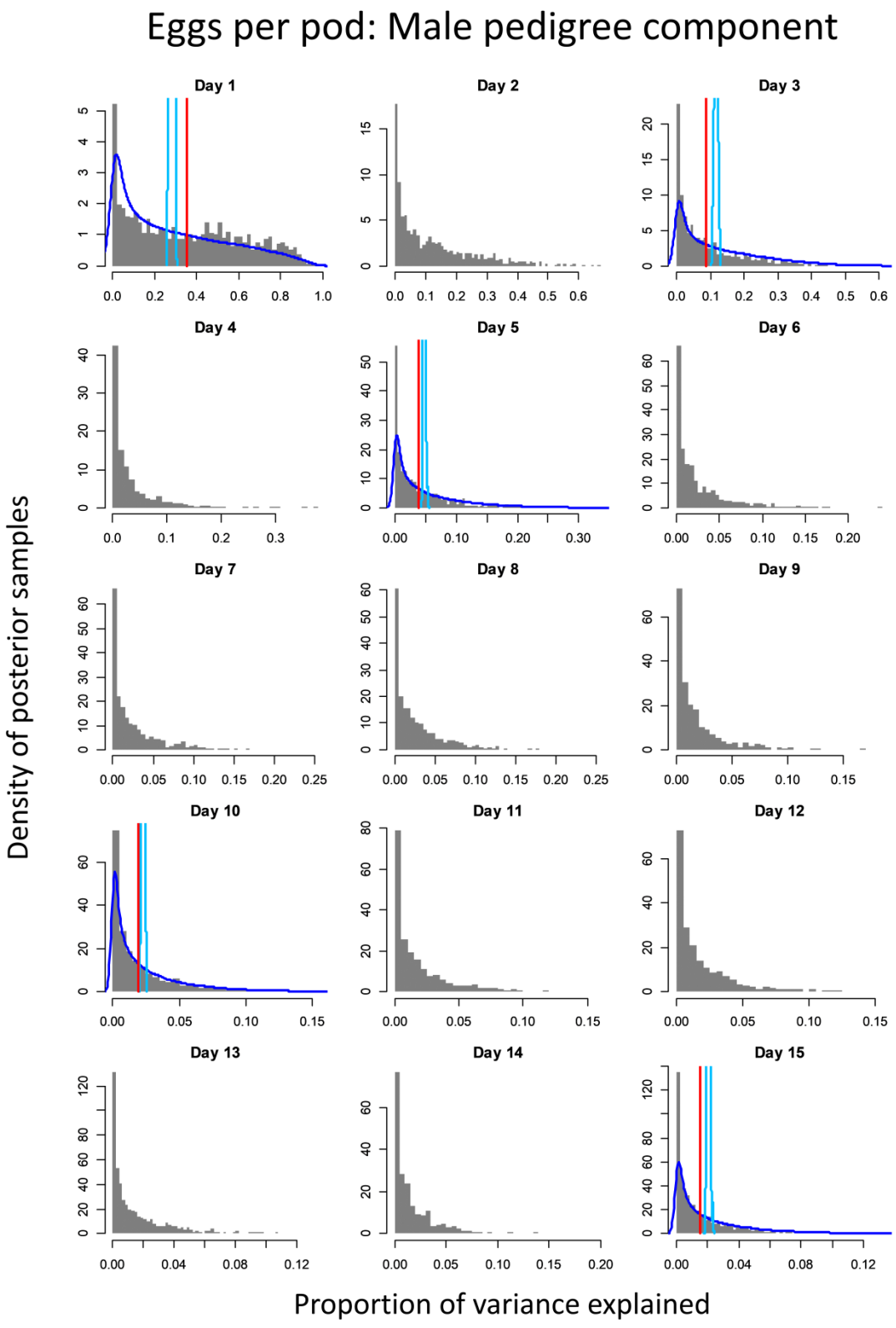


Figure S4: Posterior distributions of the male pedigree effect on number of egg pods for all subsets of the data (see main text for details on models). The light blue line shows the pooled posterior distribution of 100 independent randomizations of male pedigree links and the dark blue line shows the shows the distribution of posterior means across the 100 independent randomizations. The red vertical line shows the posterior mean for the original data. Randomization were performed for day 1, 3, 5, 10,15.

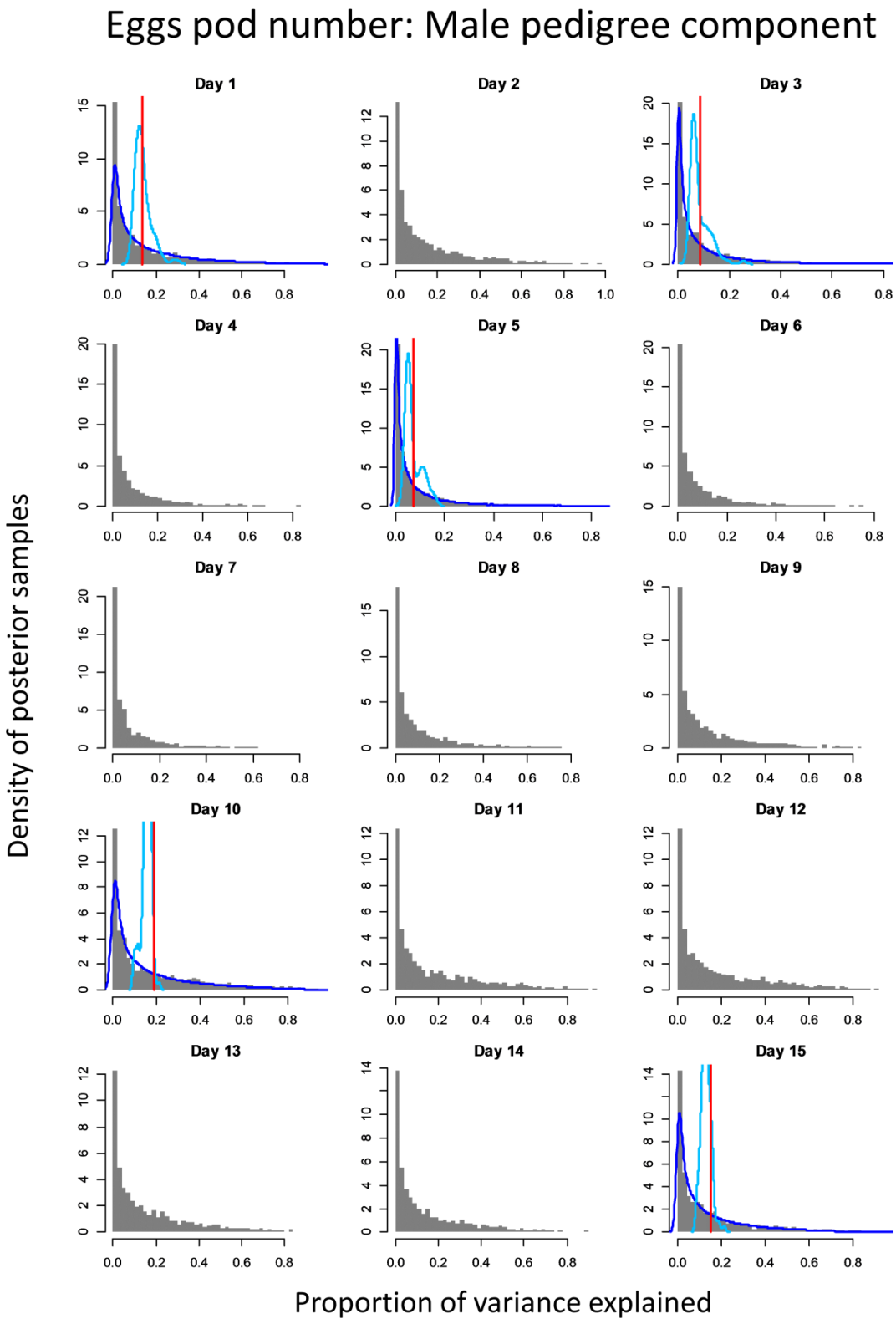


Figure S5: Posterior distributions of the male pedigree effect on latency to first egg pod (see main text for details on models). The light blue line shows the pooled posterior distribution of 100 independent randomizations of male pedigree links and the dark blue line shows the shows the distribution of posterior means across the 100 independent randomizations. The red vertical line shows the posterior mean for the original data.

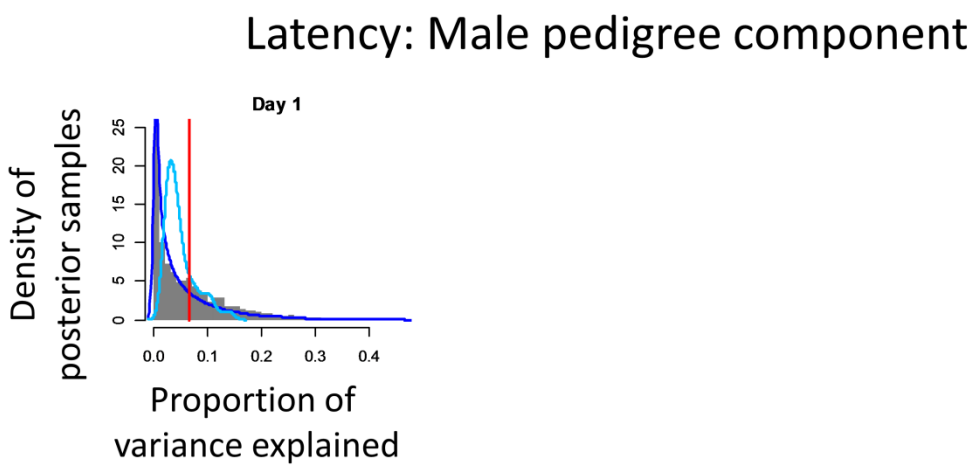


Figure S6: Posterior distributions of the female pedigree effect on egg length for all subsets of the data (see main text for details on models).

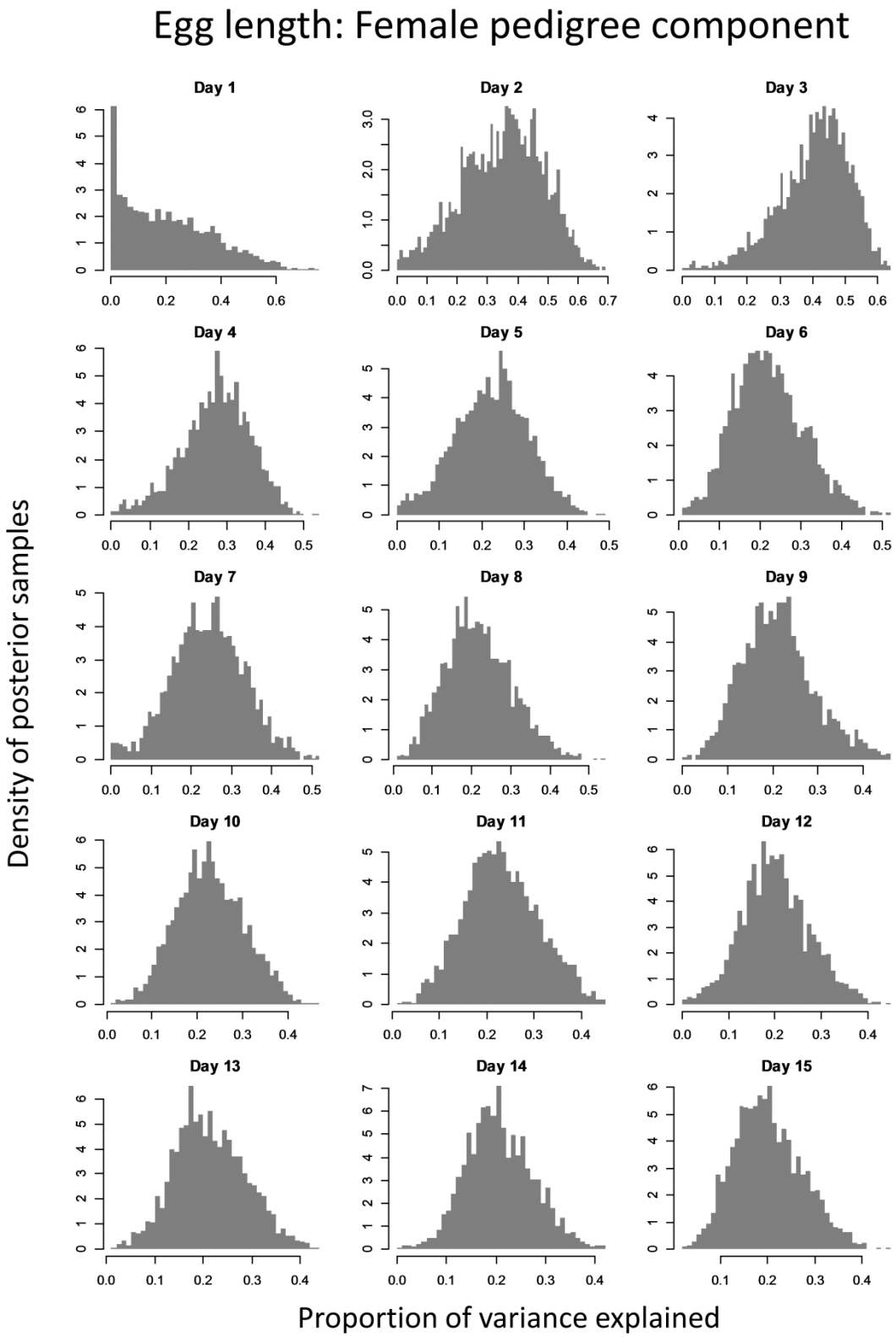


Figure S7: Posterior distributions of the female pedigree effect on egg pod length for all subsets of the data (see main text for details on models).

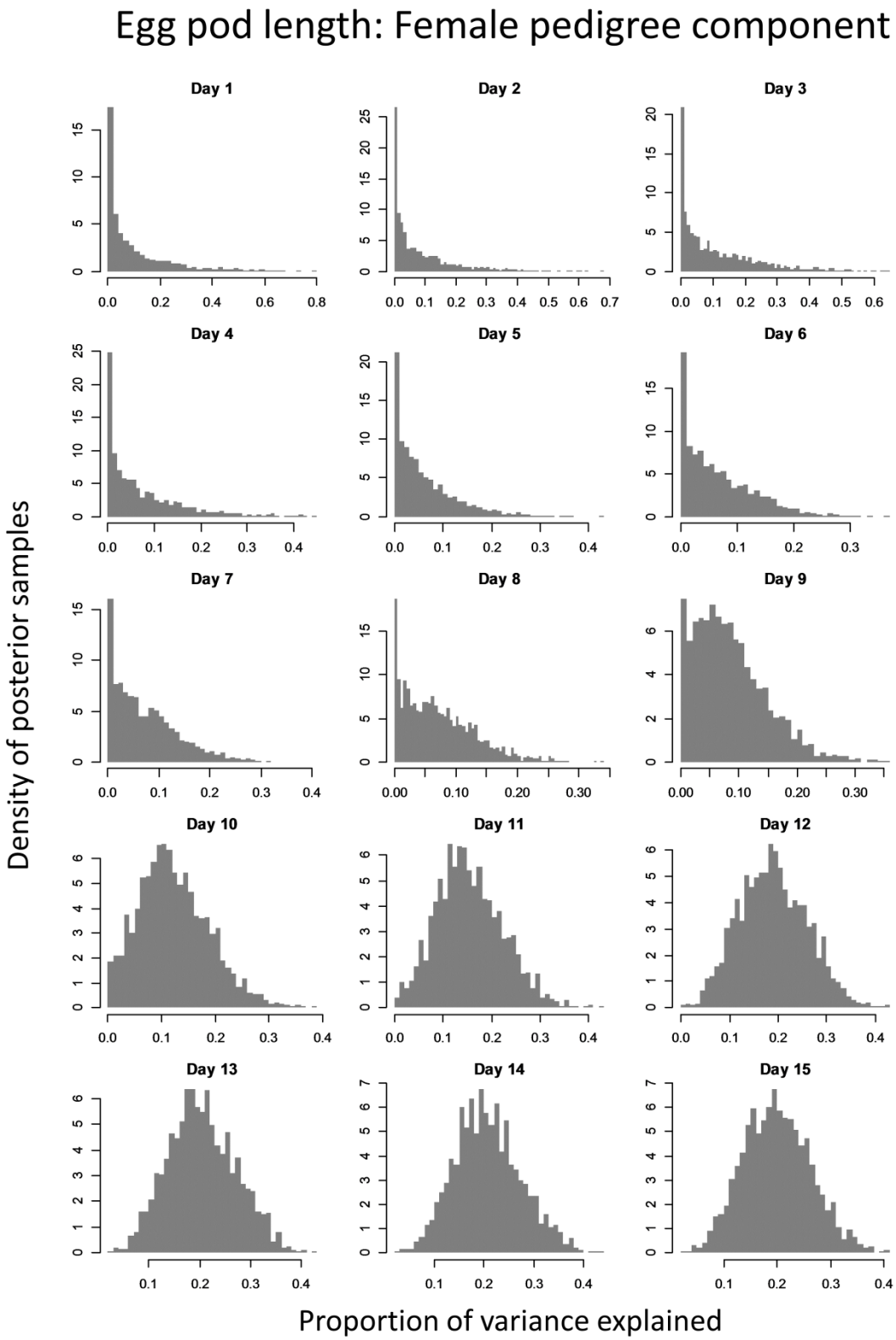


Figure S8: Posterior distributions of the female pedigree effect on eggs per egg pod for all subsets of the data (see main text for details on models).

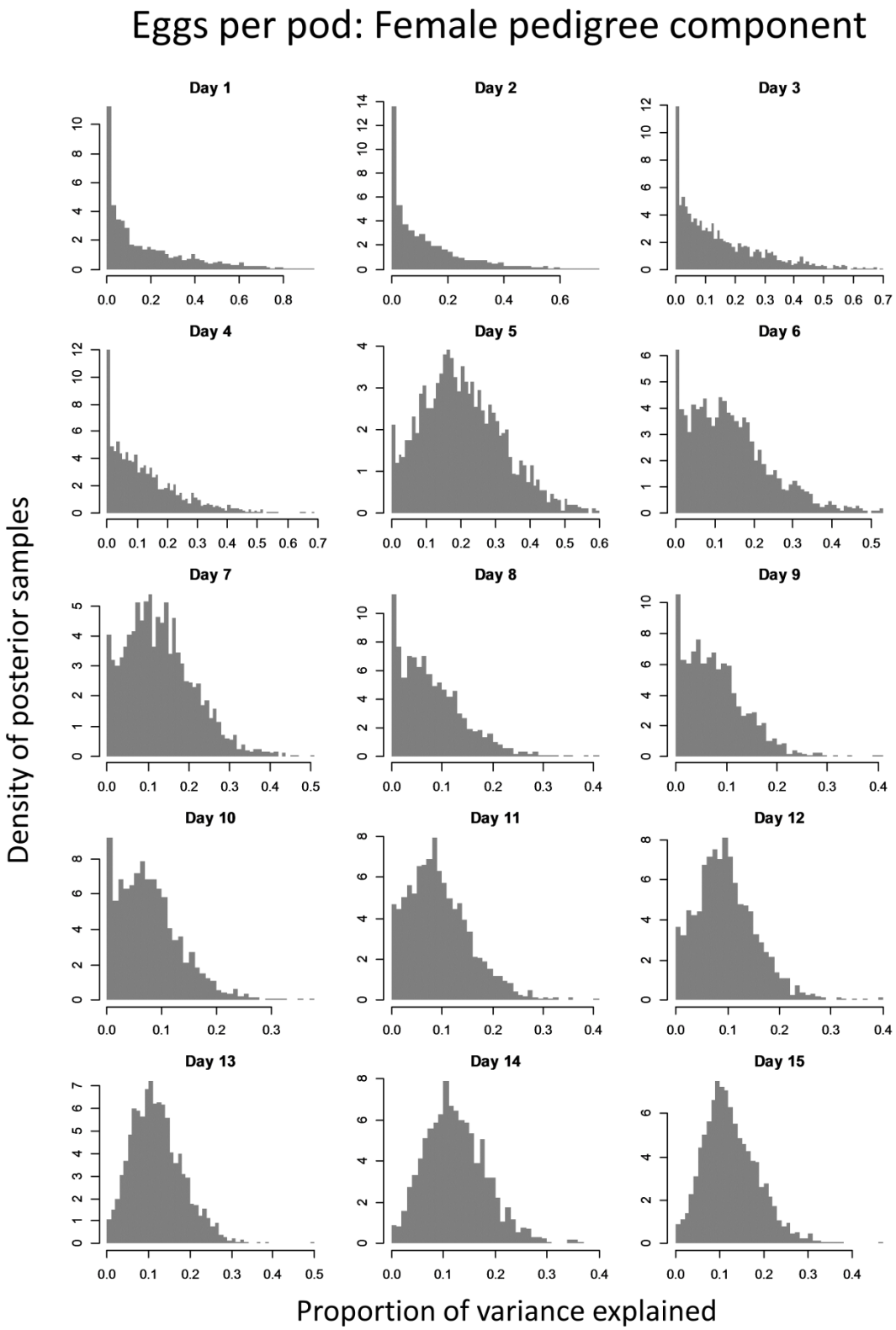


Figure S9: Posterior distributions of the female pedigree effect on number of egg pods for all subsets of the data (see main text for details on models).

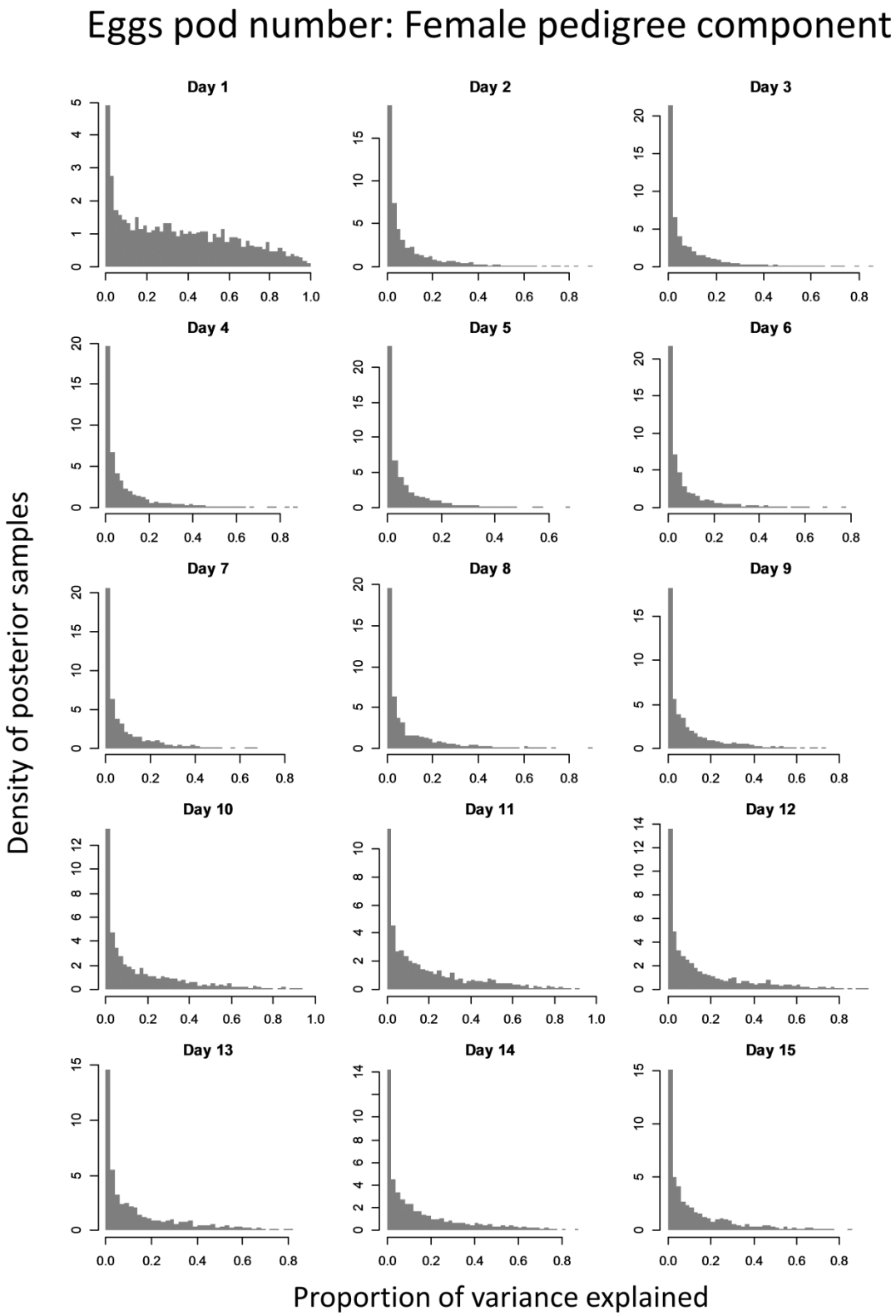


Figure S10: Posterior distributions of the female pedigree effect on latency to first egg pod (see main text for details on models).

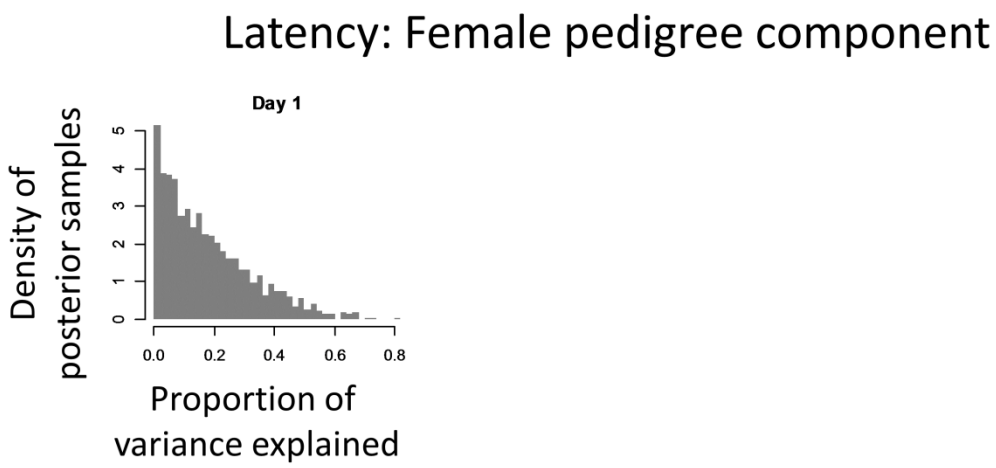


Figure S11: Variance components for a) egg length, b) egg pod length and c) number of eggs per pod across the 15-day period estimated separately across 15 datasets that (unlike in Figure 2 of the main manuscript) are limited to the data from exactly the specified day. Female additive genetic effects are shown in solid grey, male indirect genetic effect are show in in solid black, pair identity effects in coarse diagonal hatching, the cohort effect in dense diagonal hatching, the egg identity effect in coarse vertical hatching and the residual variance component in white.

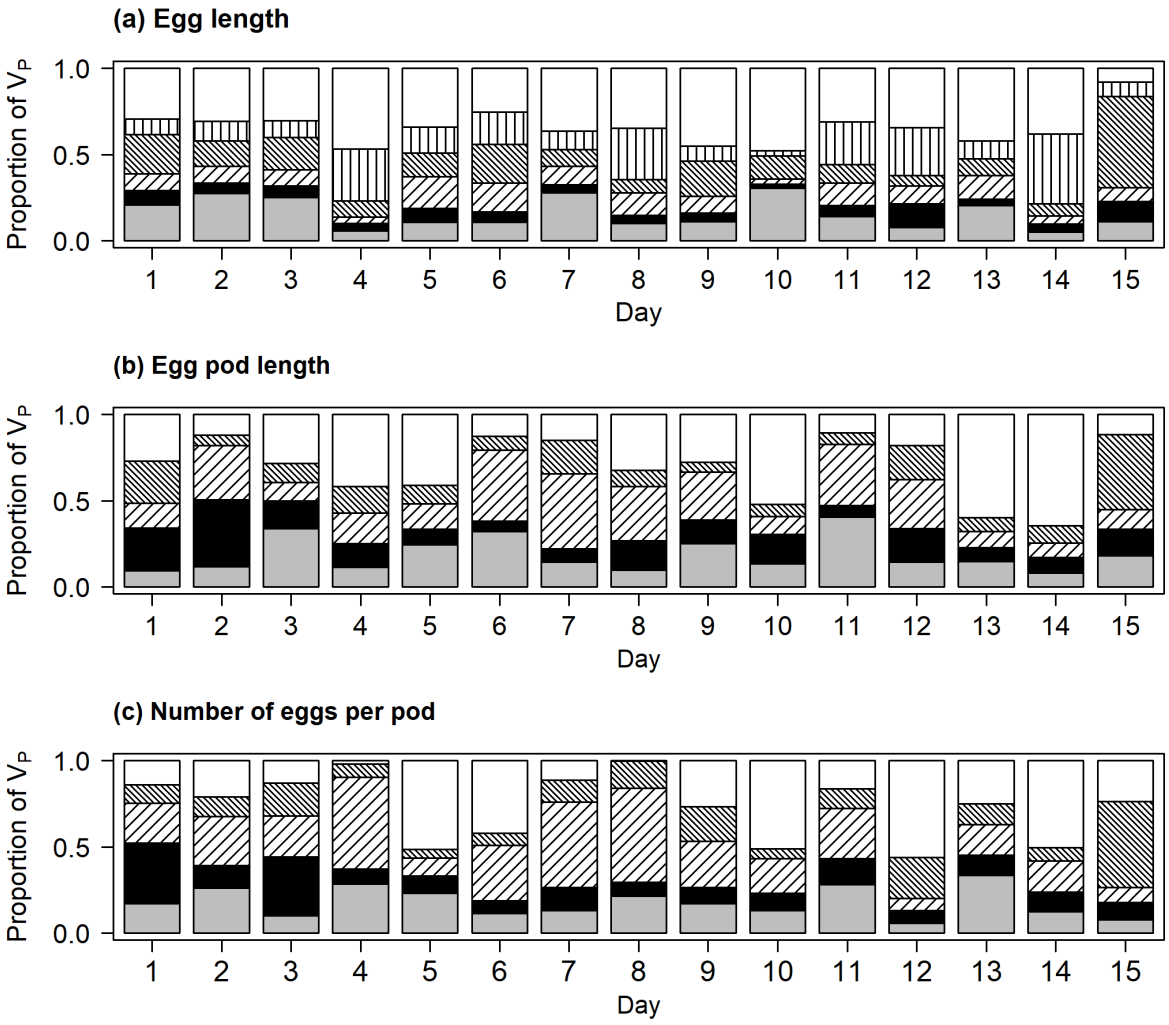
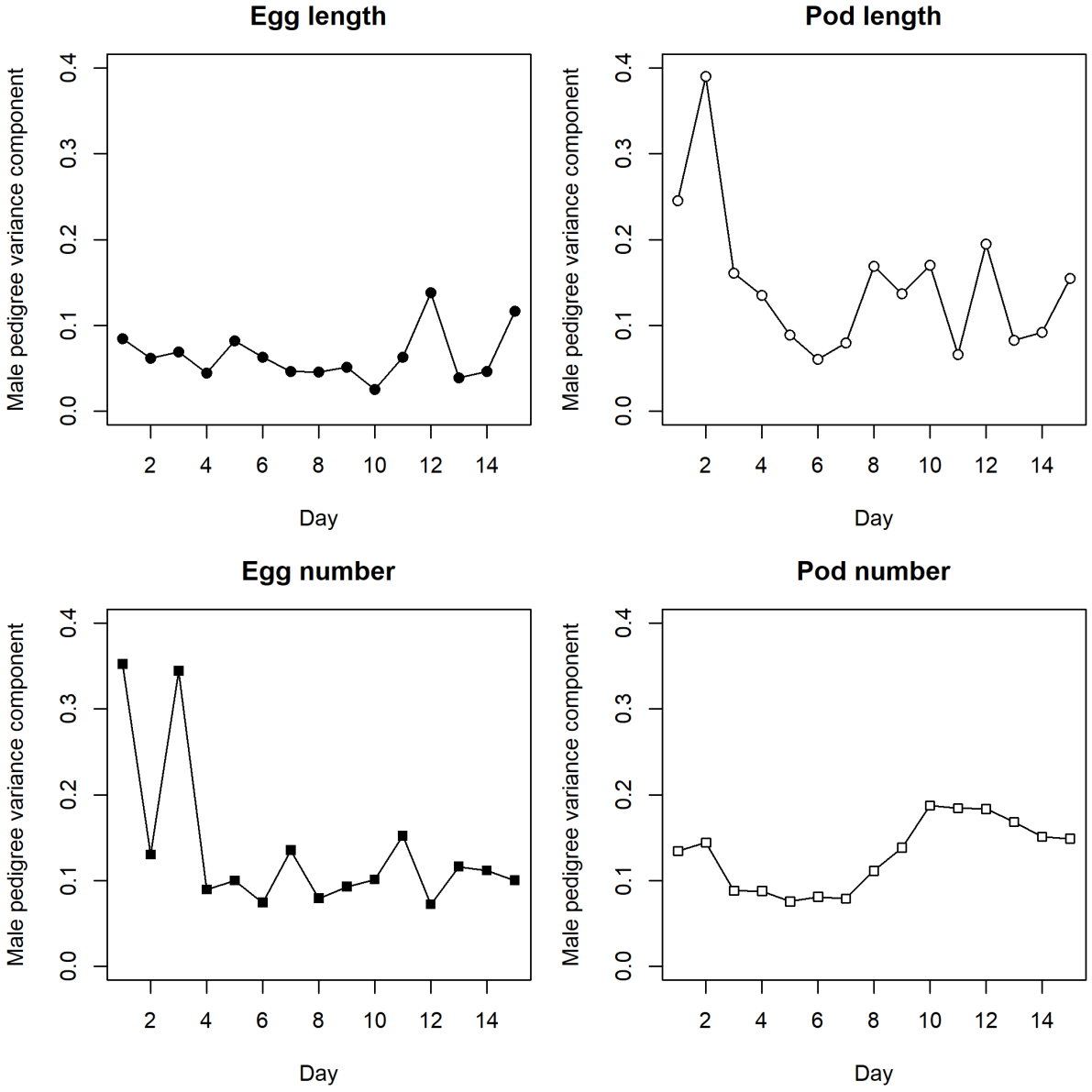


Figure S12: Temporal trend in the male indirect genetic effect variance components for a) egg length, b) egg pod length, c) number of eggs per pod and d) number of egg pods across the 15-day period. The data are from models shown in Figure S11 for egg length, egg pod length and number of eggs per pod and from Figure 2 for number of egg pods.



Declaration of Independent Assignment

I declare in accordance with the conferral of the degree of doctor from the School of Biology and Pharmacy of the Friedrich-Schiller-University Jena that the submitted thesis was only written with the assistance and literature cited in the text.

People who assisted in experiments, data analysis and writing of the manuscripts are listed as co-authors of the respective manuscripts. I was not assisted by a consultant for doctorate thesis.

This thesis has not been submitted whether to the Friedrich-Schiller-University, Jena or any other university.

Kolkata, May 27th 2020 Anasuya Chakrabarty

Declaration of Authorship

I declare in accordance with the conferral of the degree of doctor from the School of Biology and Pharmacy of the Friedrich-Schiller-University Jena that the submitted thesis is solely authored by me.

Kolkata, February 11th, 2021 Anasuya Chakrabarty